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Award Number: DAMD17-98-1-8627

TITLE: Mitochondrial Mechanisms of Neuronal Injury

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REPORT DATE: September 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2002	Annual (1 Sep 01 -31 Aug 02)

4. TITLE AND SUBTITLE
Mitochondrial Mechanisms of Neuronal Injury

5. FUNDING NUMBERS
DAMD17-98-1-8627

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
This project is focused on understanding the mechanisms of neuronal injury in acute and chronic neurodegenerative diseases. In particular, we are interested in the role that mitochondria play in both the maintenance of neuronal viability and in the execution of neurons following injurious stimuli. Our prior studies have shown that mitochondria are an essential target for calcium overload in excitotoxic neuronal injury. In the experiments described here we are pursuing the study of mitochondria in neurons in additional ways. We are developing a model of neuronal injury in an organotypic slice preparation that should provide insights into mitochondrial events in dopaminergic neurons. We hope that this will allow the study of mitochondrial physiology in neurons that are selectively vulnerable in Parkinson's disease. In addition, we are investigating the properties of mitochondrial trafficking in neurons, which, together with an in situ study of mitochondrial DNA replication, will start to provide an unprecedented insight into mitochondrial biogenesis, movement and turnover. We believe that an understanding of the long-term properties of mitochondria in neurons will provide information that is critical to the full appreciation of the mechanisms of neuronal injury in chronic neurodegenerative disease.

14. SUBJECT TERMS:	15. NUMBER OF PAGES
neurotoxin, mitochondria, neurodegeneration, excitotoxicity, apoptosis, stroke, Parkinson's disease	70
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction.

This project is focused on understanding the mechanisms of neuronal injury in acute and chronic neurodegenerative diseases. In particular, we are interested in the role that mitochondria play in both the maintenance of neuronal viability and in the execution of neurons following injurious stimuli. Our prior studies have shown that mitochondria are an essential target for calcium overload in excitotoxic neuronal injury. In the experiments described here we are pursuing the study of mitochondria in neurons in additional ways. We are developing a model of neuronal injury in an organotypic slice preparation that should provide insights into mitochondrial events in dopaminergic neurons. We hope that this will allow the study of mitochondrial physiology in neurons that are selectively vulnerable in Parkinson's disease. In addition, we are investigating the properties of mitochondrial trafficking in neurons, which, together with an in situ study of mitochondrial DNA replication, will start to provide an unprecedented insight into mitochondrial biogenesis, movement and turnover. We believe that an understanding of the long-term properties of mitochondria in neurons will provide information that is critical to the full appreciation of the mechanisms of neuronal injury in chronic neurodegenerative disease.

Body.

This project was originally funded from 9/98 until 8/01. Last year the project was continued with two supplements. One supplement was intended to allow us to develop an organotypic slice preparation that contains substantia nigra, striatum and cortex in order to use this preparation to study mitochondrial function in fully innervated dopaminergic neurons. The second supplement was provided as a part of a consortium arrangement (the consortium is directed by Dr Gary Fiskum at the University of Maryland). The project is designed to start studies of mitochondrial trafficking in neurons, and to investigate the site of mtDNA synthesis, both in primary cultures in neurons. This progress report contains a little additional information from the original project period as well a description of progress on the two supplements.

Progress on original project.

The original project was focused on understanding changes in mitochondrial function under conditions associated with neuronal injury. We published a number of papers during this project period: these are listed below but as the findings have been reported before we will not discuss them further here. We have recently submitted a manuscript that was a part of this original project period (Vergun et al, 2002). In this study we investigated the effects of hypoglycemia on the response to glutamate in primary neurons. We found that inhibition of glycolysis with 2-deoxyglucose (DOG) greatly potentiated glutamate-induced increases in intracellular calcium and exacerbated mitochondrial depolarization. By using substrates that bypassed glycolysis (pyruvate and lactate) or by inhibiting mitochondrial but not glycolytic ATP synthesis, we were able to demonstrate that either pathway could generate sufficient ATP to prevent the increase in glutamate responses. The findings of this study provide interesting additional insights

into the contributions of metabolic pathways under conditions where substrates are limited, and are thus quite relevant to ischemic brain injury. A manuscript describing the findings in detail is provided.

Progress on supplement 1: Organotypic slices.

At the inception of this project other laboratories had established the feasibility of co-culturing slices of substantia nigra (SN), striatum and cortex obtained from P1-3 rat brains (Plenz and Kitai, 1996). These studies had demonstrated that the dopaminergic neurons would grow from the SN slice into the striatum and innervate the GABAergic cells. However, as far as we are aware, the selective vulnerability of dopaminergic neurons in this preparation has not yet been demonstrated. Thus, the goals of our study were to (i) set up the preparation, (ii) establish the selective vulnerability of dopaminergic neurons, including developing methods to quantitatively measure dopaminergic cell injury, and (iii) develop techniques to allow the measurement of mitochondrial physiology from dopaminergic neurons in the slice preparation.

In a number of respects, this project has turned in to a larger challenge than initially anticipated. Nevertheless, we have made progress towards each of the goals. Firstly, we have established the culture preparation successfully. There were many issues that needed resolution, such as the appropriate conditions for cutting and attaching slices to substrates. Documenting these issues is not meaningful, but proved to be quite time consuming. Most of the year was focused on toxicity experiments. We chose 6-hydroxydopamine (6OHDA) as the first toxin to use because its' transport into dopaminergic neurons should enhance its specificity. We also used several markers to assess neuronal injury. Tyrosine hydroxylase (TH) immunohistochemistry provides a bright and reproducible marker that can identify cell bodies and processes effectively in fixed slices (Figure 1). 5,7-dihydroxytryptamine (DHT) is a fluorescent amino acid that is transported into live dopaminergic neurons. It is considerably less bright than most of the other markers but has the advantage that it can be used to identify dopaminergic neurons in live slices (Figure 2). We can also use Hoechst 33342 as a general nuclear stain to show cell presence (Figure 3), and this approach can be combined with TH staining to reveal the cellular architecture around dopaminergic cells. We have also used propidium iodide (PI) as an indicator of neuronal viability that is not selective to dopaminergic cells. PI thus indicates non-specific toxicity, and staining is typically low in healthy slices (Figure 4). We have also occasionally used other neuronal and glial markers (e.g. NeuN and GFAP). Finally, a marker of gross toxicity is the finding that the entire slice becomes detached from the substrate and is lost to further study. This is not illustrated!

Unfortunately, our experiments to this point have not clearly demonstrated selective vulnerability of dopamine neurons to 6OHDA. We have used a matrix of treatment concentrations and times to induce and assess injury. With high concentrations (5mM) for short exposure times (15min) gross injury was evident by the detachment of the slice. We evaluated lower concentrations (0.2-1mM) for short times. This typically resulted in an increase in PI staining but without a dramatic loss of TH staining (Figures 5,6) that we

anticipated with dopaminergic injury. Using 0.2mM, we extended the treatment time, and also waited longer (up to 7 days) prior to assessing injury. Once again, this approach did not result in the anticipated marked loss of TH staining, although the staining did tend to become more diffuse and less clearly associated with cell bodies (Figure 6). This alteration in the pattern of staining may be indicative of some kind of injury process. However, it is difficult to do an objective quantitative assessment of this kind of rearrangement of staining. It is also not what we expected based on studies *in vivo* and in dissociated cultures where TH staining is lost using this kind of injury paradigm. Thus, at this time we do not feel it is reasonable to conclude, based on the TH data that we are, in fact, injuring these cells.

We have tried several additional toxins. Both rotenone and MPP+ produce relatively selective dopaminergic cell injury in vivo and in dissociated cell culture. Rotenone (10nM) or MPP+ (20μM) applied for 4 days in vitro did not produce selective injury, although rotenone did produce a similarly diffuse TH staining of the slice (Figure 7). Interestingly, 24hr exposure to NMDA produced an increase in PI staining in the SN and cortex that was sensitive to MK801, demonstrating the feasibility of studying excitotoxicity in this preparation (Figure 8).

Clearly, these results were not entirely anticipated. From these experiments we have defined the following immediate goals. Firstly, we plan to use a combination of toxins in an attempt to injure the dopaminergic neurons. A sub-threshold treatment with NMDA, for example, might increase the vulnerability of the neurons. Likewise, a mild oxidative stress accomplished by the addition of BSO to deplete glutathione might also be effective. The goal of these experiments will be to deplete TH staining with minimal increase in PI beyond the appropriate region of the SN. Another possibility is that TH is a sub-optimal marker for injury, either because it is up-regulated in response to injury, or because it is cleared too slowly in this preparation. We have just initiated a series of experiments to look at other markers of injury. The dopamine transporter DAT can be labeled, for example, and the GDNF receptor c-Ret should be specific for the dopaminergic cells in this preparation. We also plan to stain for activated caspase 3, which might provide an indication of injury to cells that may not have completely died. It is also possible that the cells are, in fact, injured but have not been cleared from the slice by macrophage activity. If this were the case, the addition of microglia to the preparation might provide more evident injury.

If we can induce a compelling amount of injury, it is clearly going to be a challenge to effectively quantitate the extent of injury, because there is quite a lot of variability between the numbers of dopaminergic neurons between different slices. However, our experiments suggest that it is quite feasible to stain the cultures with DHT prior to injury, so that it is possible to get a before and after view of the cells of interest. This should limit the impact of this variability, which appears to be unavoidable with the current culturing approach.

A second goal of this project was to develop the necessary expertise to study individual neurons using fluorescence approaches in the slice. The key skill necessary here is the

introduction of dyes into identified cells, which required the use of approaches that were not initially available in our lab. However, after the purchase of a micromanipulator and pressure ejector we have been able to inject cells successfully (Figure 9). We have found that using a sharp electrode with pressure ejection, followed by the removal of the electrode, appears to be the most effective approach, and we have been able to label mitochondria in single neurons using this approach using MitoTracker Red (Figure 9) and rhodamine 123 (not shown). At this stage we have not pursued this methodology extensively in the slice preparation (although we have confirmed the lack of feasibility of bath-loading dyes into the slice) pending the successful outcome of the toxicity experiments. However, it is clear that the quality of the DHT staining is such that impaling identified cells will be quite straightforward.

Progress on supplement 2: Mitochondrial trafficking.

The second supplemental project is directed at investigating the characteristics of mitochondrial homeostasis, by which we mean the replication of mtDNA, the incorporation of new proteins, the trafficking of mitochondria, their fission, fusion and ultimately their degradation. Although all of these issues are of interest, the project concerns just two. The first topic is the characteristics of mtDNA synthesis. To investigate this we employed an in situ labeling approach described by Davis and Clayton (1996) that is based on the incorporation of BrdU into newly synthesized mtDNA. This can then be detected using a BrdU antibody (Figure 10). We have spent some time adapting this technique, and also developing image analysis algorithms that permit counting mtDNA spots in the labeled cells (Figure 11). We have been able to quantitatively determine the extent of mtDNA synthesis over time, and can detect synthesis in less than 3 hours of labeling. Thus, we are poised to investigate the questions posed in the application. For example, we are interested in determining whether injury alters the rate of mtDNA synthesis, and we would also like to establish whether mtDNA replication only occurs around the nucleus in neurons, as has been proposed to be the case in other cell types (Davis and Clayton, 1996). We are also continuing to attempt modification of this technique. As it currently stands the immunohistochemical technique destroys the other mitochondrial antigens, so it is difficult to determine where the mtDNA is in relation to the mitochondria, and thus whether all mitochondrial fragments have DNA. We hope to modify the technique to preserve some antigens, and thus be able to address this point. We also have to establish carefully that the staining we observe is not the consequence of mtDNA damage and subsequent repair, which should be possible using inhibitors of pol γ. Nevertheless, we believe this is the first in situ demonstration of mtDNA synthesis in central neurons, and this is a useful step forward in understanding mitochondrial homeostasis.

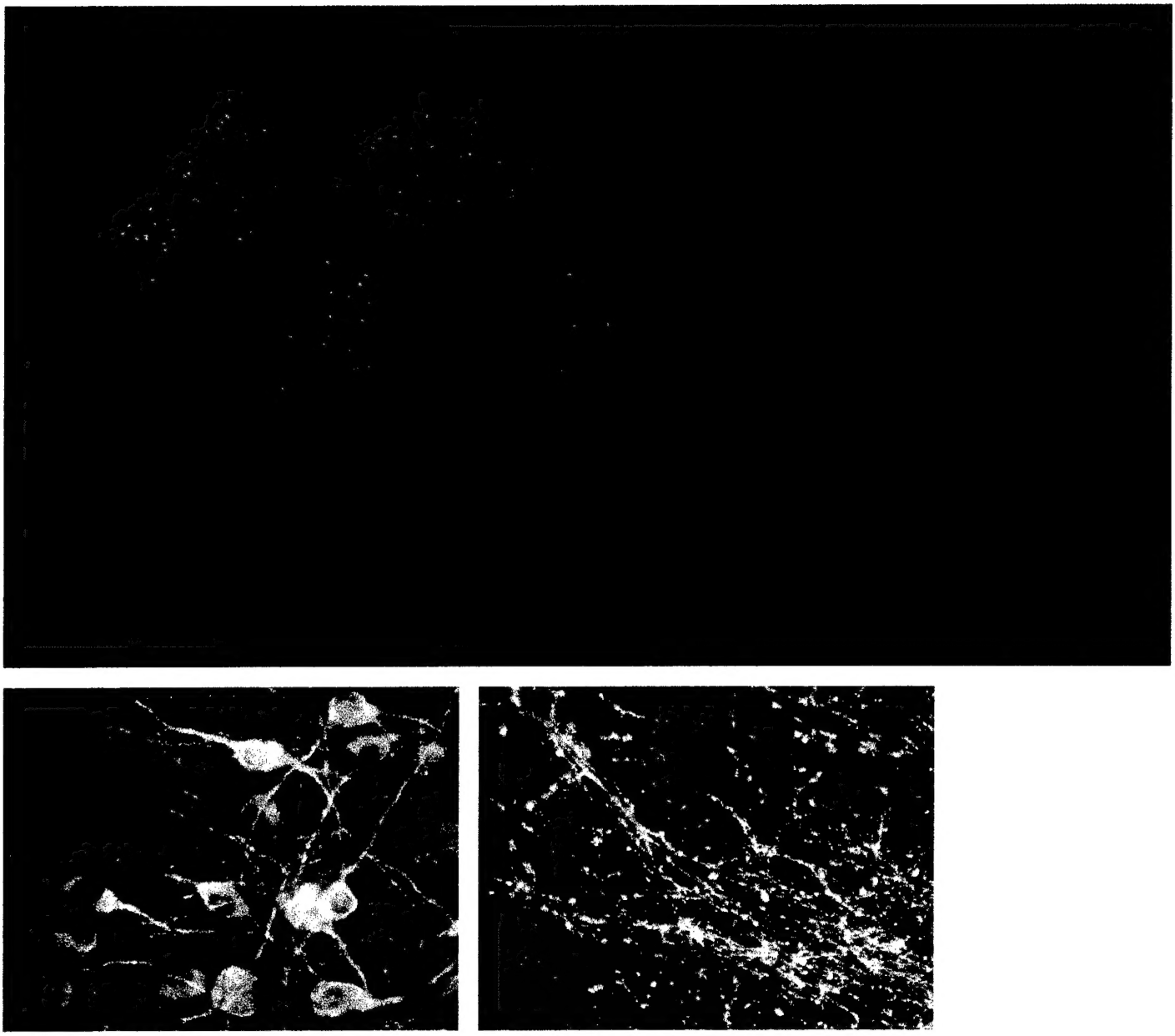
The second topic involves the investigation of mitochondrial movement in neurons and the impact of toxins on this process. We have made a good deal of progress in this aim. Firstly, we have established effective techniques for producing low efficiency transfections of primary cultures of central neurons. Using a calcium phosphate method we can reliably transfect 1-5% of the neurons with mitochondrially targeted green fluorescent proteins. Most of our studies have been done with the enhanced yellow

variant (mtEYFP), although we now have cyan and red variants available too. Perhaps more importantly, we have established methods to count movement events so that the analysis of movement can be performed objectively and quantitatively (Figure 12, 13). We have also developed imaging approaches to objectively measure mitochondrial length (Figure 14), and are implementing methods to measure the velocity of mitochondrial movement.

Using these methods we have made some interesting observations. Firstly, we have found that exposing neurons to the uncoupler FCCP substantially decreases movement of mitochondria. This is a good example of an experiment that was simply not possible prior to the introduction of these techniques, because this phenomenon could not be observed with neurons loaded with voltage sensitive dyes. Secondly, we observed that glutamate also decreased mitochondrial movement (Figure 13), but also caused a dramatic re-modeling of mitochondria (Figure 14). Both glutamate and FCCP produce effects in 1-2 minutes. The glutamate effects appear to be mediated via the activation of NMDA receptors and calcium entry, because they are mimicked by NMDA, blocked by MK801 and not mimicked by kainate, and are also prevented by calcium removal. Interestingly, the effects persist when FCCP is added together with glutamate. This suggests that (i) calcium does not have to enter the matrix to produce this effect and (ii) that remodeling is not necessarily coupled to injury, because the addition of FCCP with glutamate prevents injury (Stout et al, 1998).

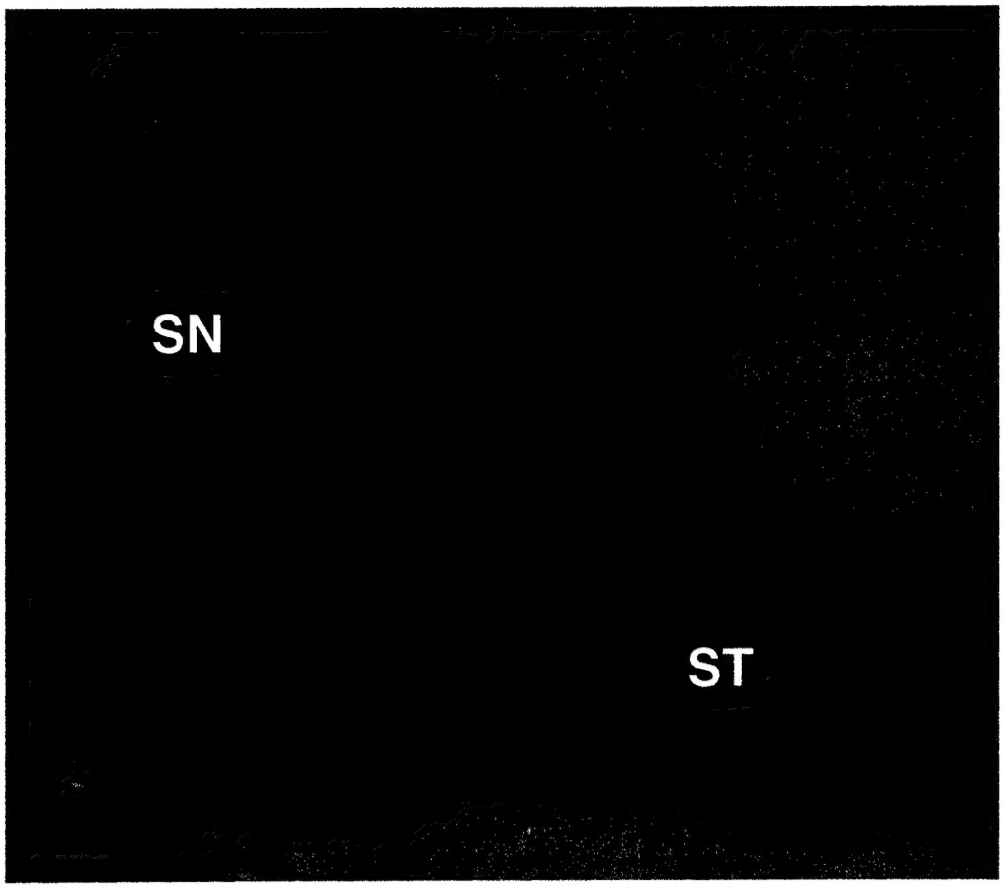
The effects of glutamate are unexpected and dramatic findings, and the observations are consistent with the notion that alterations in mitochondrial trafficking may be an important variable in neuronal injury. We are currently preparing a manuscript based on these findings, and continue to search for the mechanism that causes this effect (focused, of course, on cytoplasmic calcium mediated events). We have initiated a study of the effects of drugs that interfere with the cytoskeleton to complement these findings, and have also started to examine the effects of the additional toxins indicated in the proposal (i.e. electron transport chain inhibitors, hydrogen peroxide and zinc). At this stage it appears that all agents that depolarize mitochondria cause a decrease in movement but do not necessarily produce the rapid shape change characteristic of the glutamate response. This suggests that there are two different processes occurring – a movement effect linked to mitochondrial membrane potential in some way, and a remodeling effect mediated by calcium. We plan to continue to investigate this phenomenon in the coming year.

Figure 1



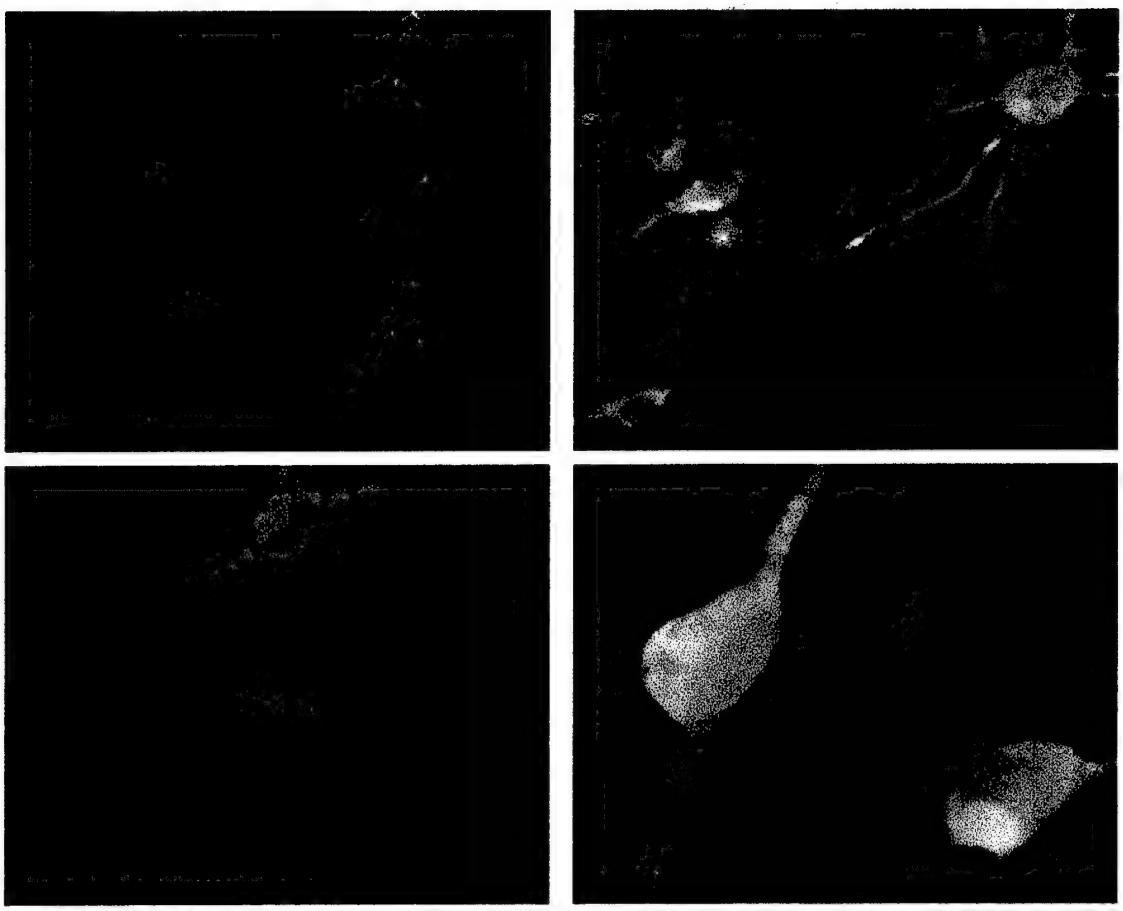
Examples of TH staining in a healthy slice. The top panel is a low-power montage of the entire slice, with the SN on the left, the striatum at center top, and the cortex at the right. The lower left image shows healthy cell bodies, while the right panel shows TH-stained fibers in the striatum.

Figure 2



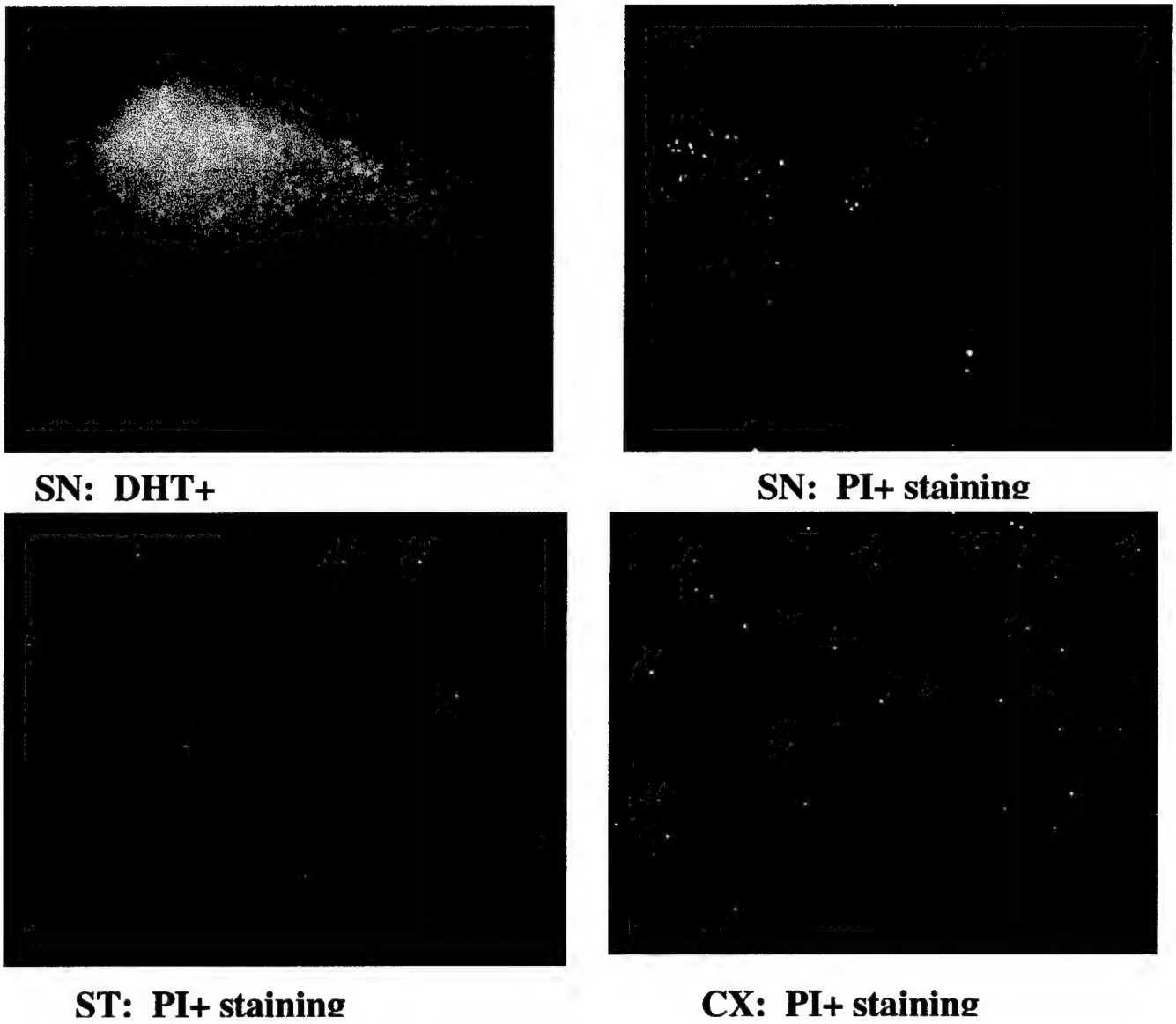
Example of 5,7-dihydroxytryptamine staining in a live slice. The dopaminergic neurons are the small bright spots in the SN.

Figure 3



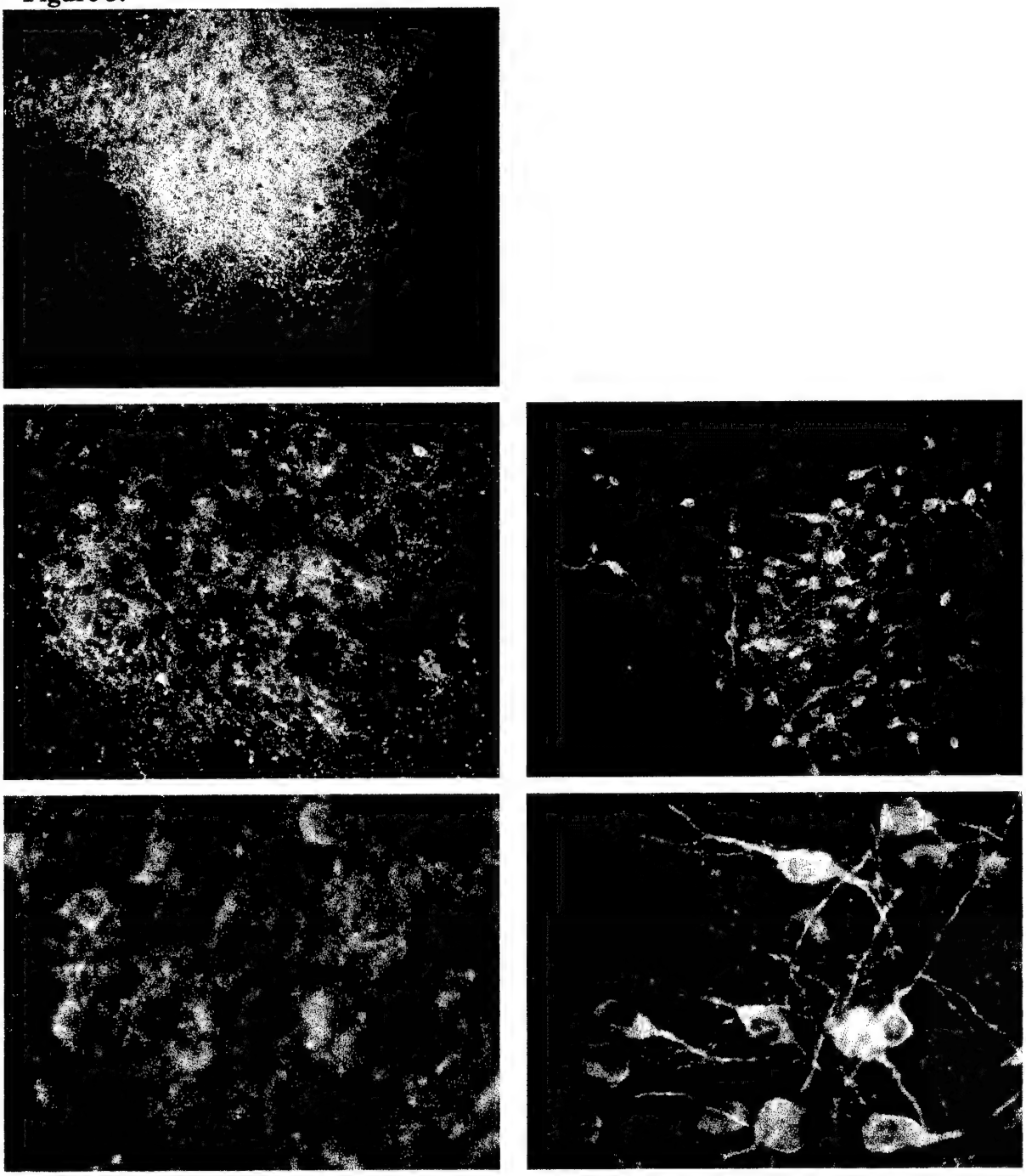
Co-labeling of slices with Hoechst 33342 and TH. On the one hand, these images illustrate the clarity with which TH positive neurons can be identified in the organotypic slice preparation. On the other, staining all the nuclei with Hoechst illustrates the complexity of the slices, too. TH staining is shown in green, while Hoechst is shown in red.

Figure 4



Propidium iodide as a tool for identifying injured neurons. In this control slice the dopaminergic neurons were identified with DHT (Top left). The slice was then labeled with PI, and the nigra (SN), striatum (ST) and cortex (CX) imaged. Note the relative paucity of PI staining in this uninjured preparation.

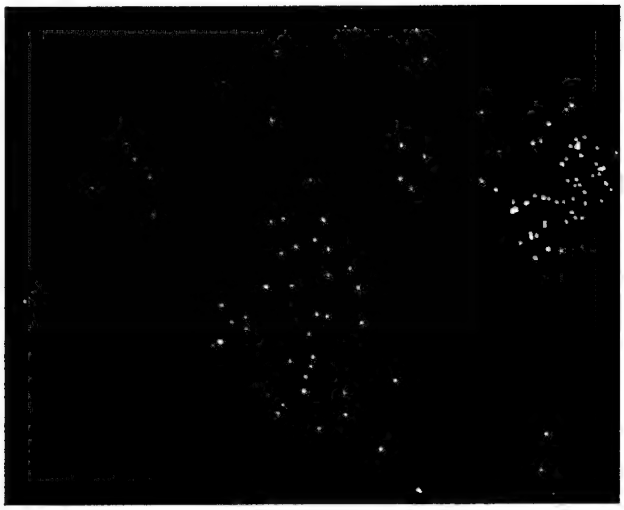
Figure 5.



TH Staining in a slice exposed to 0.2mM 6OHDA for 1hr and then maintained in culture for a further 7 days prior to fixation. The panels on the left are taken from the SN of an injured slice. The right panels are from control slices at equivalent magnification. Note the continued presence of a substantial amount of TH staining, suggesting that the dopaminergic cells have not been simply destroyed by the toxin. However, note also the loss of resolution of individual neurons and clearly defined processes, indicating that some form of damage has occurred..

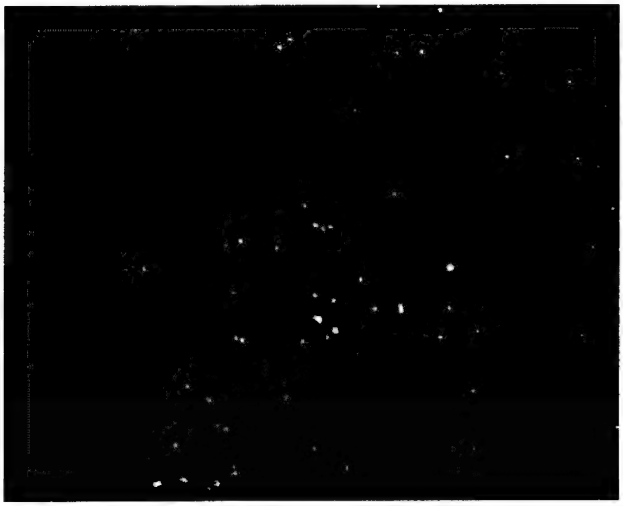
Figure 6

SN: DHT+ staining on D7



SN: PI+ staining on D7

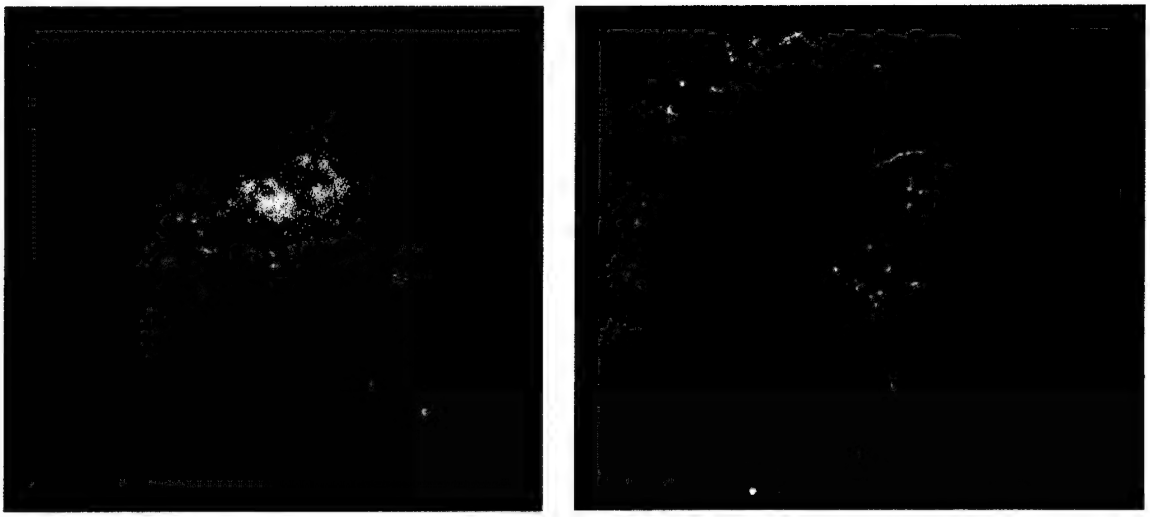
ST: PI+ staining on D7



CX: PI+ staining on D7

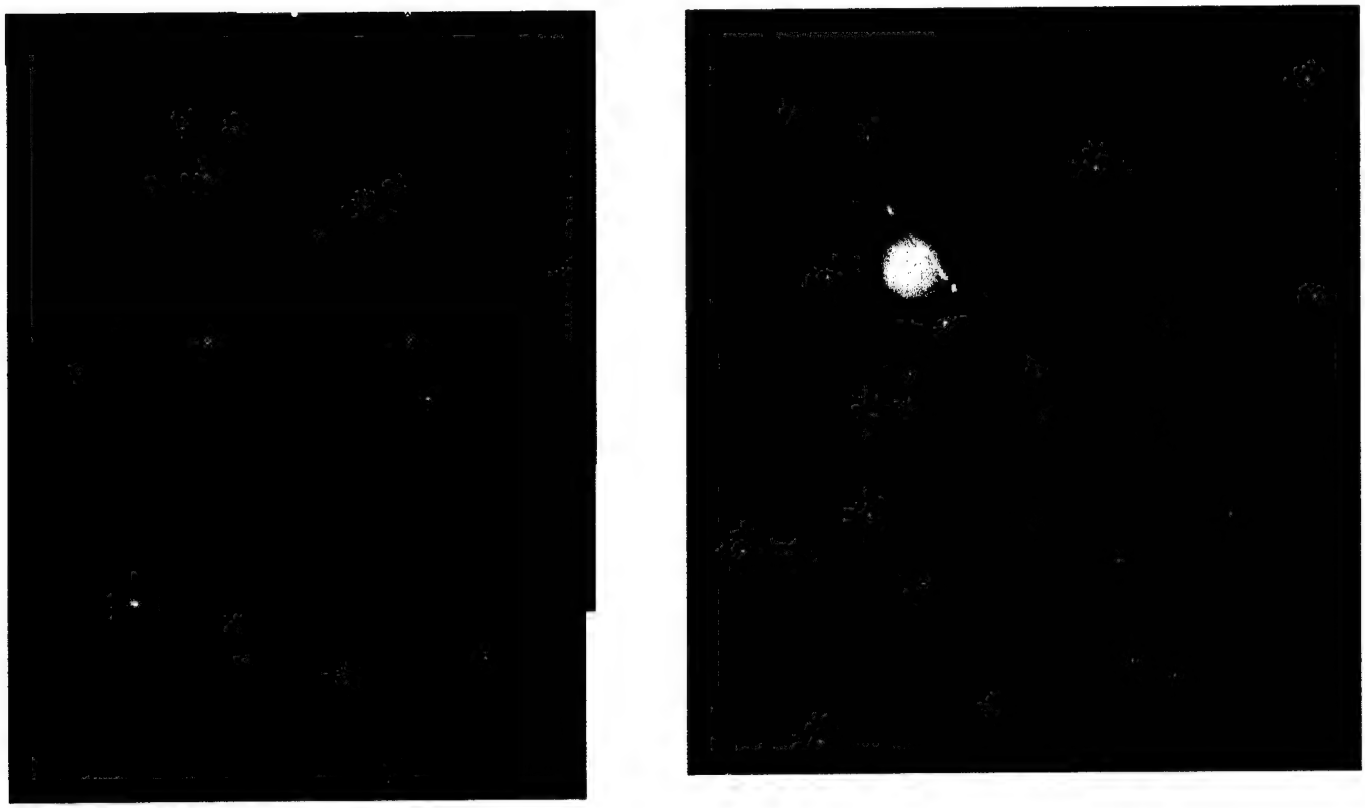
This illustration is analogous to figure 4, in that it represents DHT and PI staining of an organotypic slice preparation. This slice was exposed to 6OHDA for 1hr and these images prepared seven days later. Note the persistence of DHT staining, arguing that the dopaminergic cells are still present and expressing DAT. However, there is more PI staining evident, which we interpret as non-specific injury.

Figure 7.



Effects of other toxins. The slice on the left was exposed to 10nM rotenone for 4 days and then stained for TH. This low power image shows a similar effect to that observed with 6OHDA, in that the staining has become somewhat more diffuse and less clearly associated with cell bodies. However, the total amount of staining is still quite robust. The slice on the right was exposed to 25μM MPP+ for 4 days prior to staining. There is less evidence for diffuse staining in this slice, but there are clearly TH positive cells that remain intact after this treatment. Higher concentrations resulted in a marked increase in PI staining (not shown).

Figure 8



Excitotoxicity in the slice preparation. The slice on the left was exposed to 0.3mM NMDA with 0.01mM glycine for 24hr and then stained with PI. Note an abundance of PI positive cells. This image is of the SN region of the slice. On the right, the slice was exposed to the same treatment except that the non-competitive NMDA antagonist MK801 (10μM) was included in the incubation. The SN is outlined for ease of identification. Very few cells are PI positive in this case. This demonstrates that it is possible to produce and detect receptor-mediated injury in this preparation.

Figure 9
Introduction of dyes into individual neurons via a microelectrode. The left panel is a phase image with the electrode in place. The electrode contained 1mM MitoTracker Red in an intracellular solution. The middle panel is a fluorescence image immediately after the electrode was withdrawn. The right panel was taken five minutes later, and clearly shows more widely distributed punctuate structures that are likely to be mitochondria.

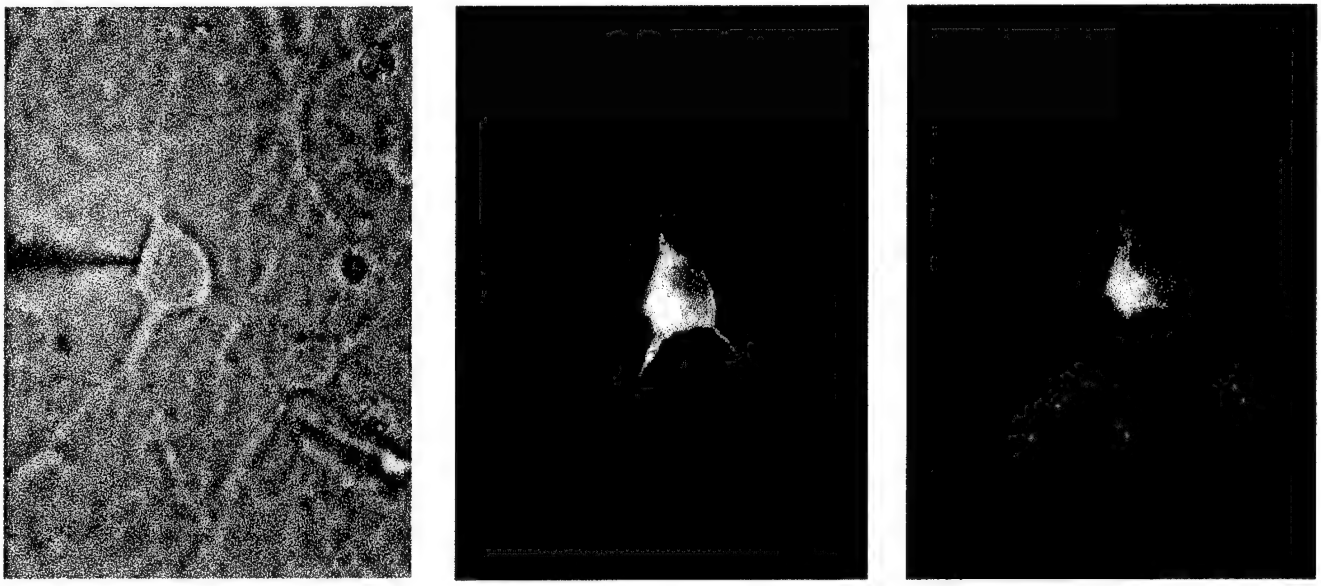
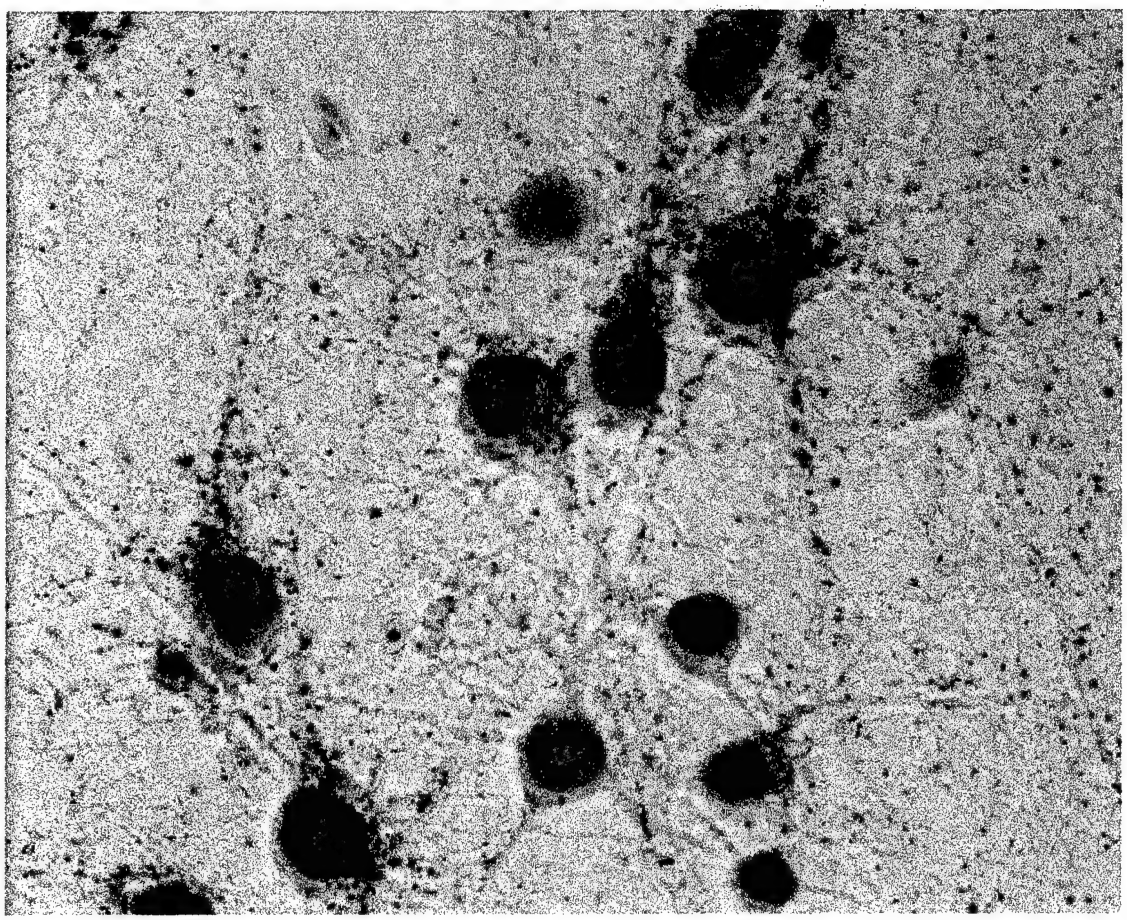
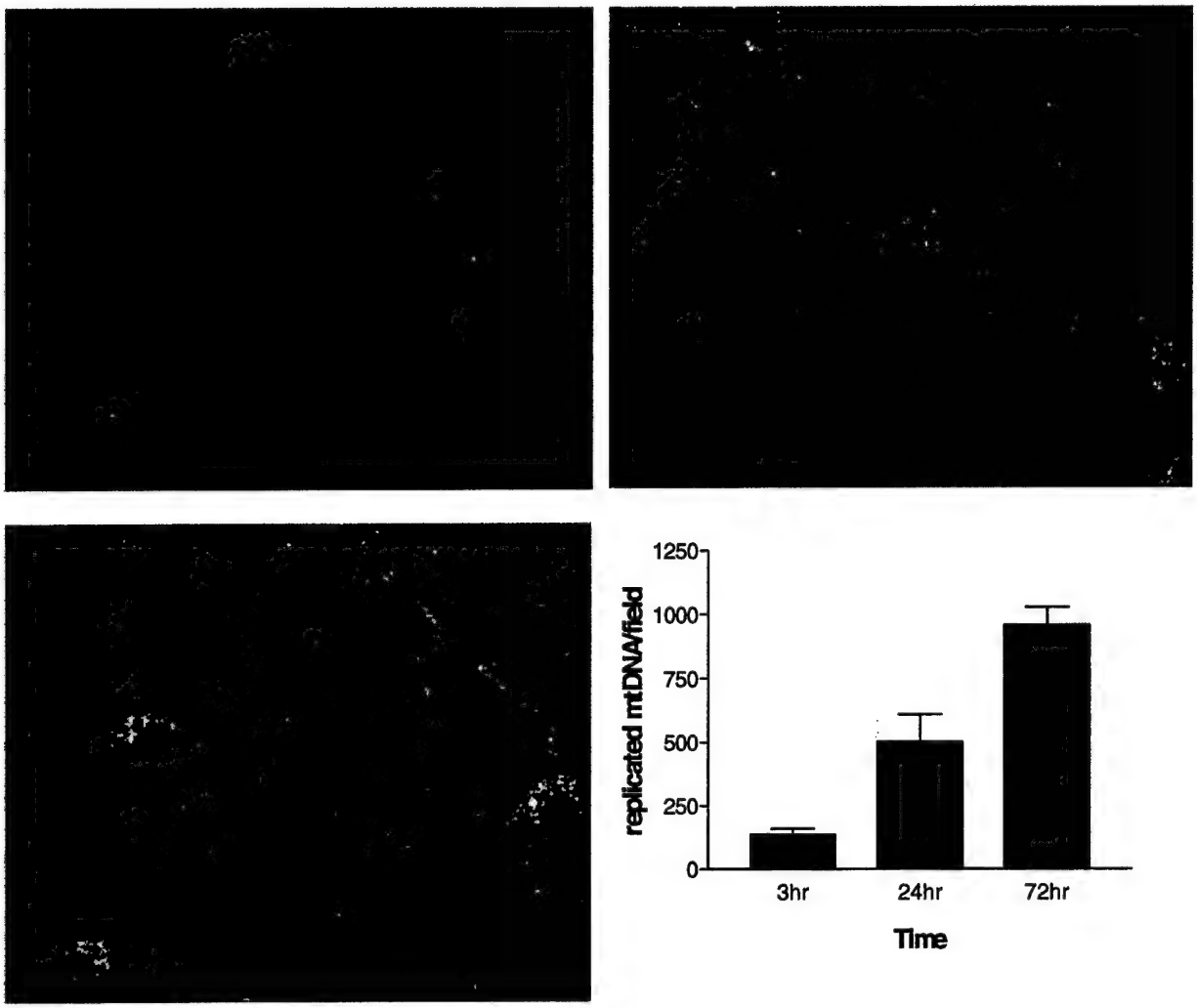


Figure 10



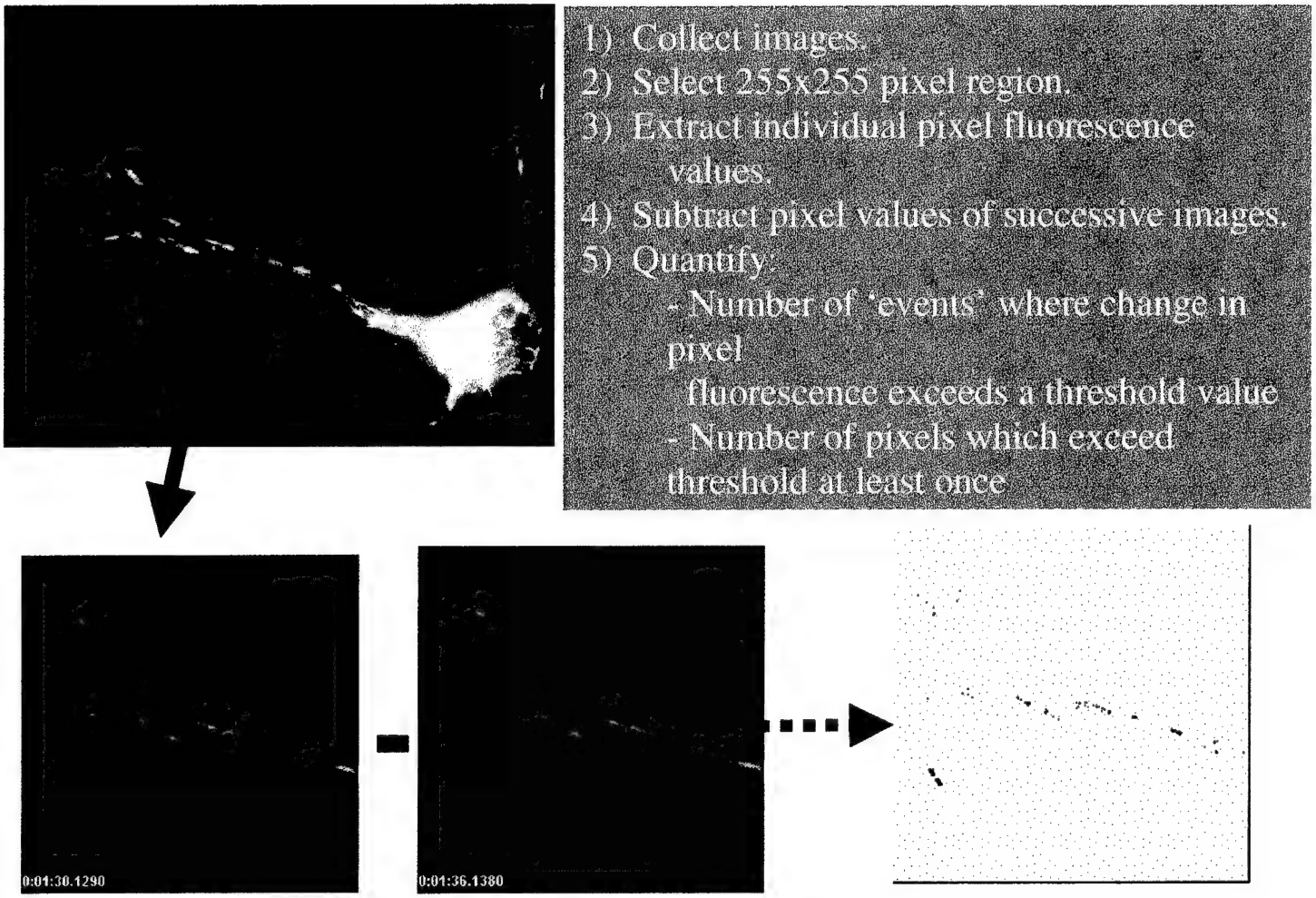
Identification of newly synthesized mtDNA in situ in primary neuronal cultures. The red spots represent BrdU incorporated into the mitochondrial genome, identified immunohistochemically. The blue represents nuclei counterstained with Hoechst 33342, and the fluorescence images are overlaid on a DIC image of the same field. In this experiment cells were loaded with BrdU for 24hr.

Figure 11.



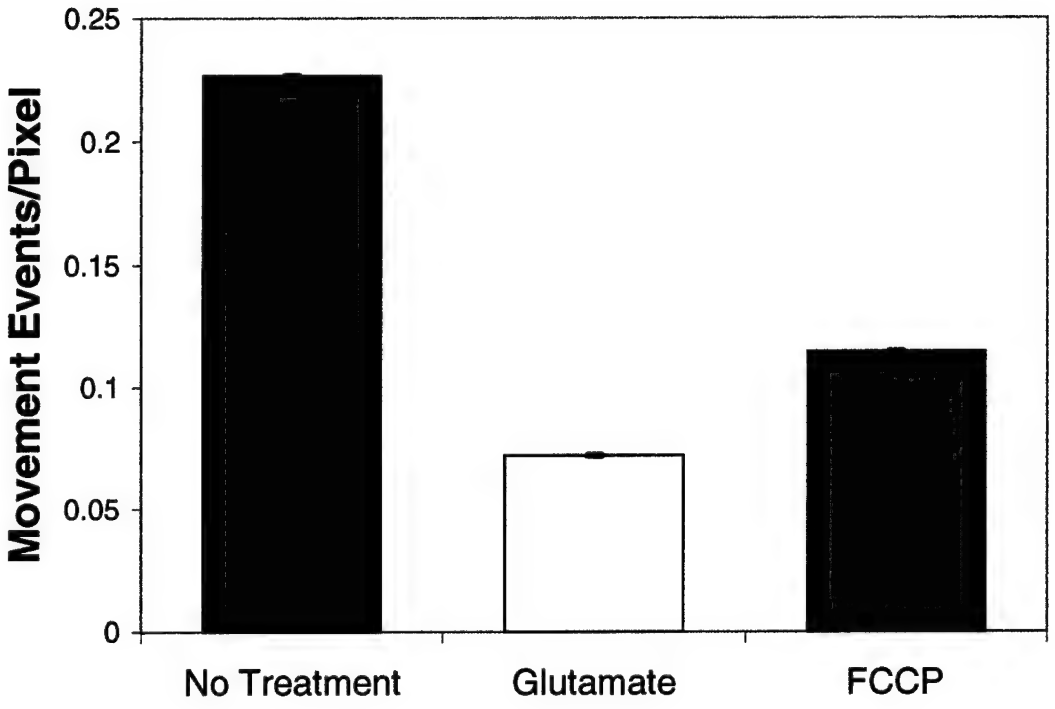
Quantitative determination of BrdU incorporation. These images show BrdU incorporation as a function of time, and demonstrate the feasibility of using image analysis to count the spots of mtDNA. The bar chart represents the mean +/- SEM of 3-8 separate experiments.

Figure 12.



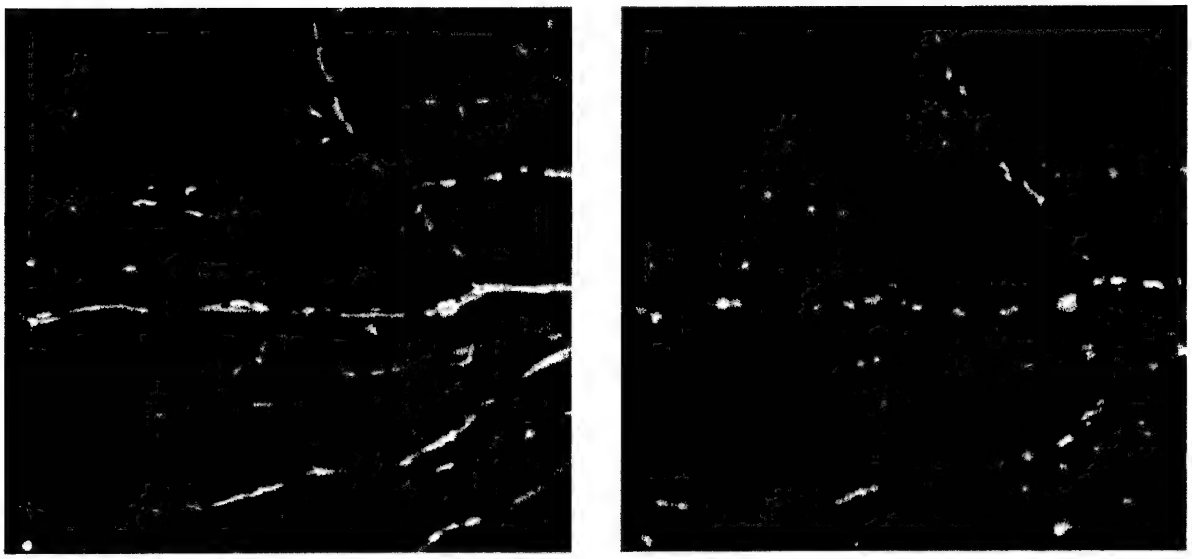
Scheme for quantitative analysis of mitochondrial movement. This approach uses an image subtraction method for comparing adjacent images in movies to detect objects that have moved, and counts each pixel that changes intensity as a single event.

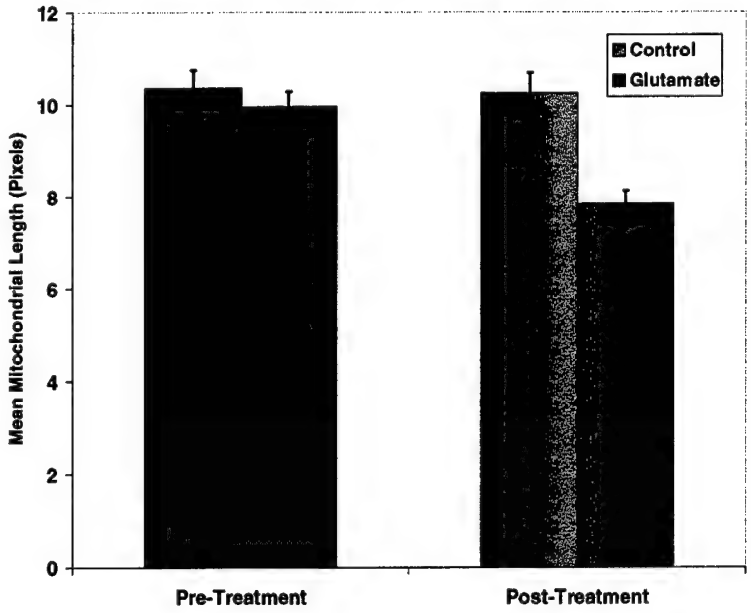
Figure 13.



Glutamate and FCCP decrease mitochondrial movement. Using the method shown in figure 12, movement was detected before and after treatment with either 30μM glutamate or 750nM FCCP. The decreases in movement are significant in each case.

Figure 14.





Effects of glutamate on mitochondrial shape. The top left panel shows mtEYFP-labeled mitochondria before glutamate application, while the right panel was taken from the same field approximately 3 minutes after the addition of 30μM glutamate. The bar chart represents the mean pixel length of about 2000 objects before and after glutamate treatment, and shows a significant decrease in mean pixel length. Note that the mean length statistic is skewed somewhat by the number of short objects that are detected in control as well as treated images.

Key Research Accomplishments.

During this project period we have:

1) Established an organotypic slice preparation that includes substantia nigra, striatum and cortex, and established that dopaminergic neurons from the SN innervate the striatum and cortex.

2) Developed techniques for introducing dyes into neurons using microelectrodes

3) Established methods for the first in situ demonstration of mitochondrial DNA synthesis in neurons in primary culture

4) Established methods for measuring mitochondrial shape and movement in neurons in primary culture

5) Discovered that FCCP and glutamate stop mitochondrial movement in neurons

6) Discovered that glutamate produces an NMDA- and calcium-dependent remodeling of mitochondria in neurons.

Reportable Outcomes.

The following list reflects all of the reportable outcomes over the duration of this project. The manuscript indicated by an asterisk is included in the appendix.

The paper indicated by * is included in the appendix.

The following papers have been published:

Berman, S.B., Watkins, S.C. and Hastings, T.G. Quantitative biochemical and ultrastructural comparison of mitochondrial permeability transition in isolated brain and liver mitochondria: evidence for reduced sensitivity of brain mitochondria. Exp. Neurol. 164:415-425 (2000).

Hoyt, K.R., McLaughlin, B.A., Higgins, D.S. and Reynolds, I.J. Inhibition of glutamate-induced mitochondrial depolarization by tamoxifen in cultured neurons. J. Pharmacol. Exp.Ther. 293 480-486 (2000).

Reynolds, I.J and Hastings, T.G. The role of the permeability transition in glutamate-mediated neuronal injury. In: Mitochondria and pathogenesis, Lemasters, J.J. and Nieminen, A.-L. (Eds), Plenum Press, New York. (2001).

Brocard, J.B., Tassetto, M. and Reynolds, I.J. Quantitative evaluation of mitochondrial calcium content following an NMDA receptor stimulation in rat cortical neurones. J. Physiol. **531**:793-805 (2001).

Scanlon, J.M., Brocard, J.B., Stout, A.K. and Reynolds, I.J. Pharmacological investigation of mitochondrial Ca^{2+} transport in central neurons: studies with CGP-37157, an inhibitor of the mitochondrial Na^{+}/Ca^{2+} exchanger. Cell Calcium. **28**:317-327 (2000).

Buckman, J.F. and Reynolds, I.J. Spontaneous changes in mitochondrial membrane potential in cultured neurons. J. Neurosci. **21**: 5054_5065 (2001).

Votyakova, T.V. and Reynolds, I.J. $\Delta\Psi_m$ - Dependent and independent production of reactive oxygen species by rat brain mitochondria. J. Neurochem. **79**:266-277 (2001).

*Vergun, O., Han, Y.Y., and Reynolds, I.J. Glucose deprivation produces a stable increase in sensitivity to glutamate in cultured rat cortical neurons. Submitted to J. Neurochem. (2002).

We have also published abstracts (about 12) in the volumes associated with the Society for Neuroscience annual meeting, the American Society for Neurochemistry and the recent meeting of the New York Academy of Sciences on Parkinson's Disease.

Conclusions.

This project is focused on the role of mitochondria in neuronal injury. While our prior studies have focused on acute changes in mitochondrial function in neurons in dissociated culture, the project is now heading in new directions. The first of these directions involves studying longer-term aspects of mitochondrial function. Specifically, we are interested in the mechanism of mitochondrial biogenesis and trafficking, because we believe that the effective delivery of mitochondria to critical regions in neurons may be as important as the function of mitochondria when they arrive there. The studies we have reported here represent several advances towards that general goal. It is clearly possible to monitor mitochondrial movement in neurons both qualitatively and quantitatively, and also make objective measurements of movement and size. Moreover, even our initial experiments have uncovered a fairly substantial impact of injury on both movement and size of mitochondria, indicating that there may be an as yet unappreciated interface between signaling events and mitochondrial trafficking. Our attempts to measure a different parameter of mitochondrial homeostasis, namely mtDNA synthesis, has also been successful, so we are effectively developing an armamentarium of techniques with which to investigate the broader issues of mitochondrial homeostasis in neurons.

The second major direction is the study of mitochondria in identified neurons. A key characteristic of all neurodegenerative diseases is the loss of selected populations of neurons. This is a feature that is notably absent in dissociated cell culture. We have elected to develop the nigro-striatal-cortical culture model to enable us to study

dopaminergic neurons that accurately innervate their relevant targets. Ultimately, we hope this will provide a model system in which we can study mitochondrial events relevant to Parkinson's disease. There are clearly a number of mostly technical obstacles to overcome before this goal can be met. We have addressed some of those during this project period, and have effectively established the model in the laboratory. However, it has proven difficult to demonstrate selective injury to the dopaminergic neurons in this culture, even though the toxins that we have employed are well-characterized. Nevertheless, we have been able to demonstrate excitotoxicity in this model system, and we are much closer to being able to objectively characterize changes in dopaminergic neurons with the toxins used thus far. We have also developed the techniques in the lab to allow us to perform imaging experiments in this preparation, and have thus at least partially met the goal of the project period.

These new approaches offer a great deal of promise for future studies. We are already in the position to gain an unprecedented look into the life history of the mitochondrion in the neuron, and we expect to be able to do this in specifically identified neurons in the not too distant future. This will allow us to determine just what the mitochondria are doing and when they are doing it. Ultimately, we will be able to provide an important description of the behavior of a critical contributor to neuronal injury, and possibly suggest novel targets for therapeutic intervention in neurodegenerative disease.

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Vergun, O., Han, Y.Y. and Reynolds, I.J. (2002) Glucose deprivation produces a stable increase in the sensitivity to glutamate in cultured rat cortical neurons. Submitted.

Glucose deprivation produces a stable increase in sensitivity to glutamate in cultured rat cortical neurons

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Abbreviations: $\Delta\Psi_m$, mitochondrial membrane potential; $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration; $[Na^+]_c$, cytosolic Na^+ concentration; Rh123, rhodamine 123; DOG, 2-Deoxy-*D*-glucose; FCCP, carbonyl cyanide *p*-trifluoro-methoxyphenyl hydrazone; DNP, dinitrophenol; HBSS, HEPES-buffered salt solution; TTX, tetrodotoxin.

Running title: Glycolytic impairment and calcium homeostasis

Key words: mitochondria, excitotoxicity, glutamate, glycolysis, ATP, 2-deoxy-*D*-glucose

Abstract

In this study we investigated whether the link between mitochondrial dysfunction and deregulation of Ca^{2+} homeostasis preceding of excitotoxic cell death is mediated by the cellular deenergization. Glycolytic and/or mitochondrial ATP synthesis were inhibited with 2-deoxy-*D*-glucose (DOG) and oligomycin, and the changes in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) and mitochondrial membrane potential ($\Delta\Psi_m$) were measured in response to small sub-toxic (5-10 μM) glutamate concentrations. $[Ca^{2+}]_c$ and $\Delta\Psi_m$ were simultaneously measured in individual neurons using the fluorescence dyes fura-2FF and Rhodamine 123. DOG, which blocks glycolysis and also inhibits mitochondrial respiration due to depletion of pyruvate, greatly increased and accelerated glutamate-induced rise of $[Ca^{2+}]_c$ and mitochondrial depolarization. These DOG-induced changes were very stable; a washout of DOG up to 150 minutes with glucose-containing medium did not reduce the effect. Prior blockade of only glycolytic (DOG with pyruvate) or only mitochondrial (oligomycin) ATP synthesis did not increase the response of neurons to glutamate. These studies show that to maintain the sensitivity of neurons to glutamate at control levels at least one of the cellular sources of ATP production must be be intact. Either glycolysis or oxidative phosphorylation can effectively support Ca^{2+} homeostasis in cultured forebrain neurons.

Introduction

Pathologic elevation of extracellular glutamate concentration during hypoxic-ischemic brain injury induces protracted stimulation of NMDA receptors and leads to a sustained increase in $[Ca^{2+}]_c$ (Choi and Rothman 1990). It is widely believed that a disturbance of calcium homeostasis in neurons triggers a series of pathological processes leading to delayed neuronal death (Schanne et al. 1979; Choi 1988; Ogura et al. 1988; Manev et al. 1989; Choi and Rothman 1990; Glaum et al. 1990; deErausquin et al. 1990; Limbrick et al. 1995). However, the exact mechanism of the irreversible neuronal calcium overload in response to the overstimulation of glutamate receptors is still under investigation. The possibilities include an enhanced Ca^{2+} entry across the plasma membrane (Manev et al. 1989) and an impairment of Ca^{2+} extrusion from the cytoplasm both out of the cell (Na^+/Ca^{2+} exchanger, Ca^{2+}-pump) and into mitochondria (for review see Khodorov, 2000). It is known that Ca^{2+} overload of neurons, correlating with glutamate excitotoxicity, is coupled with mitochondrial Ca^{2+} uptake and mitochondrial depolarization (Ankarcrona et al. 1995; Budd and Nicholls, 1996a; Isaev et al. 1996; Khodorov et al. 1996; Shinder et al. 1996; White and Reynolds 1996; Scanlon and Reynolds 1998; Stout et al. 1998; Kiedrowski 1998; Vergun et al. 1999; Ward et al. 2000). Profound mitochondrial depolarization reverses the mitochondrial ATP synthase, which both suppresses mitochondrial ATP production and promotes ATP hydrolysis (Nicholls 1986; Leyssens et al. 1996; Duchen 1999). Prolonged exposure of neuronal cultures to glutamate reduces the intracellular ATP content (Tsuji et al. 1994; Marcaida et al. 1995; Budd and Nicholls 1996a). Because the major neuronal Ca^{2+}-extrusion systems, the plasma membrane Ca^{2+} pump and the Na^+/Ca^{2+} exchanger require ATP (DiPolo and

Beauge 1988; Carafoli 1991; Sheu and Blaustein 1992), a failure of Ca^{2+} extrusion could result from cytoplasmic ATP depletion. This concept has been supported by Budd and Nicholls (1996a, b) who have shown that deregulation of $[Ca^{2+}]_c$ in cerebellar granule cells in response to glutamate or KCl appears under conditions where the cellular ATP/ADP ratio is decreased. A good correlation between the glutamate-induced decrease in ATP content and neuronal degeneration in cultured spinal neurons has also been reported (Tsuji at al. 1994).

On other hand, there is evidence that the depletion of cytoplasmic ATP mediated by activation of kainate and NMDA receptors is not directly correlated with neuronal death (Marcaida et al., 1995). Moreover, growth factors could protect against excitotoxic/ischemic damage without prevention of ATP depletion (Mattson et al. 1993a,b), and an increase in [ATP] by glutamate antagonists is unrelated to neuroprotection (Riepe et al. 1994).

In order to investigate the role of cellular energy production in neuronal Ca^{2+} overload and mitochondrial dysfunction we examined the effect of inhibition of mitochondrial and/or glycolytic ATP synthesis on the neuronal responses to relatively low, non-toxic concentrations of glutamate. Our results show that blockade of only glycolytic ATP synthesis or only mitochondrial ATP synthesis did not increase neuronal responses to glutamate. A suppression of both glycolytic and mitochondrial ATP synthesis produces a sustained increase in sensitivity of the neurons to glutamate. These data show that either glycolysis or oxidative phosphorylation can effectively support Ca^{2+} homeostasis in forebrain neurons.

Materials and Methods

General Materials. All raw materials and reagents were purchased through Sigma (St Louis, MO), unless otherwise specified.

Cell culture. All procedures using animals were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee. Cultures of forebrain neurons were prepared as described previously (Brocard et al., 2001). In brief, forebrains from embryonic day 17 Sprague Dawley rats were removed and dissociated with trypsin. Cells were plated on poly-D-lysine-coated 31 mm glass coverslips and inverted after 24 hr to decrease glial growth. Neurons were cultured in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, 24 U/ml penicillin, 24 μg/ml streptomycin; final glutamine concentration, 3.9 mM. Experiments were performed when cells were 12-14 days in culture.

Experimental Paradigm. Unless otherwise specified, all experiments were performed using HEPES-buffered salt solution (standard HBSS) with the following composition (in mM): NaCl 137, KCl 5, $NaHCO_3$ 10, KH_2PO_4 0.6, Na_2HPO_4 0.6, $MgSO_4$ 0.9, $CaCl_2$ 1.4, HEPES 20, and glucose 5.5 (pH adjusted to 7.4 with NaOH). For experimental conditions requiring glucose deprivation, the HBSS solution was devoid of glucose (glucose-free HBSS). Coverslips containing neuronal cells were pre-incubated for 60 minutes at 37°C (concurrent to fluorescent dye loading; see below) in HBSS containing various inhibitors

of glycolytic and/or mitochondrial ATP synthesis. The cells were then washed for 20 to 25 minutes at room temperature (~25°C) in standard HBSS (containing glucose). The coverslips were then carefully placed into a perfusion chamber that was then mounted onto a microscope fitted for fluorescence imaging and measurements. Fluorescence measurements were performed at room temperature while the cells were perfused at 10 ml/min with HBSS and were then exposed to glutamate for 10 minutes. All experiments were repeated 3-7 times using cultures from different culture dates. Controls for each experiment were performed on sister cultures

Fluorescence measurements of $[Ca^{2+}]_c$ and $\Delta\Psi_m$. For $[Ca^{2+}]_c$ measurements, cells were incubated for 60 minutes at 37°C with 5 μM fura-2FF-AM (Teflabs, Austin, TX) and 5 μg/ml bovine serum albumin in HBSS. For simultaneous measurements of $[Ca^{2+}]_c$ and $\Delta\Psi_m$, 5 μM Rh 123 (Molecular Probes, Eugene, OR) was added to the medium during the last 15 minutes of the fura-2FF incubation period. The cells were then washed with HBSS and placed into perfusion chamber on a BX50WI Olympus Optical (Tokyo, Japan) microscope fitted with an Olympus Optical LUM PlanFI 40x water immersion quartz objective. $[Ca^{2+}]_c$ and $\Delta\Psi_m$ were monitored in single cells using excitation light provided by a 75 W xenon lamp-based monochromator (T.I.L.L. Photonics GmbH, Martinsried, Germany). Cells were alternatively illuminated with 340 and 380 nm light for fura-2FF and 490 nm for Rh123. Emitted fluorescence was passed through a 500 nm long pass dichroic mirror and a 535±40 nm band pass filter (Omega Optical). Fluorescence data was acquired and analyzed using Simple PCI software (Compix Inc, Cranberry PA). Fluorescence was measured in 15-25 individual neurons for each coverslip. Background

fluorescence, determined from three or four cell-free regions of the coverslips, was subtracted from all the signals prior to calculating the ratios. Accumulation of Rh 123 in polarized mitochondria quenches the fluorescence signal; in response to mitochondrial depolarization the dye redistributes throughout the cell and fluorescence emission is dequenched. An increase in Rh 123 signal therefore indicates mitochondrial depolarization (Duchen and Biscoe, 1992). Because Rh 123 is a single wavelength dye and the signal therefore varies between the cells, all Rh 123 data have been normalized between minimum and maximum intensity. In all experiments the maximum Rh 123 signal was obtained following complete dissipation of $\Delta\Psi_m$ with FCCP (750 nM) at the end of the experiment.

Statistics. Statistical analysis was performed using Prism 3.0 (Graph Pad Software, San Diego CA). All the data are presented as mean ± S.E.M. Comparisons were made using Student's t test, with P values of less than 0.05 taken as significant.

Results

Glutamate-induced increase in $[Ca^{2+}]_c$ and mitochondrial depolarization .

Application of glutamate (in the presence of glycine) to 12-14 DIV cortical neurons induced an increase in $[Ca^{2+}]_c$ and a concurrent depolarization of mitochondrial membrane (Figure 1, A). These responses typically consisted of a rapid, initial peak with a following secondary rise in $[Ca^{2+}]_c$ to a plateau. The mitochondrial depolarization usually followed the plateau. The time from the onset of the glutamate stimulus to the secondary large rise in $[Ca^{2+}]_c$ and mitochondrial depolarization varied considerably between individual neurons from seconds to minutes, but usually did not exceed 10 minutes.

Application of high (30-100 μM) glutamate concentrations induced secondary increases in $[Ca^{2+}]_c$ and mitochondrial depolarization that were essentially irreversible and did not return to the resting levels during a 15-20 minutes glutamate washout period (Figure 1A). Similar relationships between $[Ca^{2+}]_c$ and $\Delta\Psi_m$ were described before in hippocampal cultured neurons (Vergun at al, 1999; 2001; Keelan at al. 1999).

Application of lower (5-10 μM) glutamate concentrations produced smaller increases in $[Ca^{2+}]_c$ and more modest mitochondrial depolarization. With these lower concentrations the large, secondary change was observed in 37% of cells (Figure 1B, n=40 experiments), and the time to the secondary change was typically longer (see below). In the other 63% of the neurons we did not observe the secondary change during the 10 minutes of glutamate exposure.

A loss of $[Ca^{2+}]_c$ homeostasis after overstimulation of glutamate receptors and excessive Ca^{2+} influx into the cytosol is correlated with glutamate-induced neurotoxicity (see Choi & Rothman, 1990) and believed to be central to the process of excitotoxicity. One of the possible mechanisms responsible for the deterioration of Ca^{2+} homeostasis is a decrease in intracellular [ATP] due to mitochondrial depolarization. In order to investigate this hypothesis we suppressed cellular ATP production by blockade of glycolytic and/or mitochondrial ATP synthesis.

Glucose deprivation-induced increase in sensitivity to glutamate.

Substitution of glucose in HBSS with 10 mM DOG, a glucose analog that is phosphorylated but not further metabolized, induced a small mitochondrial depolarization (Figure 2, B) with no increase in baseline $[Ca^{2+}]_c$ (measured with Fura-2FF). However, 40-60-minutes of glucose deprivation (with addition of DOG) greatly enhanced the neuronal responses to subsequent, prolonged (10 min) application of glutamate. Figure 2, B shows the changes in $[Ca^{2+}]_c$ and $\Delta\Psi_m$ induced by 10 μM glutamate in DOG-treated cells, while figure 2A shows controls from sister cultures. In this group of cultures glutamate failed to induce the second phase of Ca^{2+} and mitochondrial responses in 38% of the control neurons (n=3 experiments); the development of the second phase in the rest 62% of the cells was with about 500 second delay. However, in DOG-treated cells the response to glutamate was large and rapid. Glucose deprivation decreased both the number of the neurons with a small and reversible response to glutamate and the time to maximum of mitochondrial depolarization. The differences in response magnitude and delay are summarized in figure 2C.

The response of the cells to glutamate remained enhanced after a 10-150 minutes washout of DOG. Thus, in subsequent experiments we incubated neurons in DOG-containing, glucose-free buffer for 1h at 37°C during the dye loading period, and then returned the neurons to glucose-containing HBSS for 20-25 minutes at room temperature prior to imaging. Using this approach, the small DOG-induced mitochondrial depolarization recovered to baseline prior to the start of the recording. Glutamate was then applied for 10 minutes. Figure 3A-B shows an example illustrating these experiments, and the summarized data are presented in figure 3C. Clearly, preincubation with DOG produces a profound enhancement in the sensitivity to glutamate in these neurons. Figure 4 shows a similar increase in $[Ca^{2+}]_c$ responses of the neurons following DOG exposure, but this time using repeated brief exposures to glutamate. Figure 4A illustrates the changes in $[Ca^{2+}]_c$ in the response to 1.5 minute stimulus of 10 μM glutamate in control cells. After the cells were deprived of glucose (with addition of 10 mM DOG) for 60 minutes with a following 25 minutes washout with normal HBSS, $[Ca^{2+}]_c$ responses of the neurons were greatly enhanced (Figure 4, B). In some cells after the second and third stimulus $[Ca^{2+}]_c$ reached a plateau level and did not recover, while in other cases the recovery was greatly delayed. Note that the baseline ratio of fura-2FF in the cells that were deprived of glucose had not changed from the control level, showing that basal calcium homeostasis remained intact in this paradigm.

It is possible that the effects of DOG could be mediated by an acute modulation of glutamate receptors. To test this possibility we applied 20 mM DOG against a background of a small concentration of glutamate (Figure 5). DOG did not further increase $[Ca^{2+}]_c$, meaning that this compound has no direct action on glutamate receptors,

and that the effect of DOG is not associated with an enhanced Ca^{2+} influx into the cytosol. We also found that a longer (5 h) incubation of cells in glucose-free medium without addition of DOG has similar effect to DOG (data not shown), consistent with the notion that the primary effect of DOG is a consequence of the inhibition of glycolysis.

It has been shown that preventing of mitochondrial Ca^{2+} uptake during stimulation of NMDA receptors significantly enhanced increases in $[Ca^{2+}]_c$ and significantly reduced glutamate-stimulated neuronal cell death (Stout at al, 1998). It is possible that mitochondria accumulated Ca^{2+} during glucose deprivation so that enhanced susceptibility of the neurons to glutamate is due to mitochondrial Ca^{2+} overload. Depolarization of mitochondria with an uncoupler (FCCP) can be used as a method of estimating the accumulation of Ca^{2+} in the mitochondria of neurons (Brocard at al., 2001). In figure 1B and 3A it is clearly seen that application of 750 nM FCCP in the end of the experiments induced release of mitochondrial calcium. However, application of FCCP to cells prior to glutamate exposure induced only a small increase in $[Ca^{2+}]_c$ (Figure 6, A). Glucose deprivation did not enhance FCCP-induced increase in $[Ca^{2+}]_c$ (Figure 6, B), indicating that mitochondria did not take up Ca^{2+} during incubation of the cells in glucose-free DOG-containing medium, and that increased sensitivity of glucose deprived neurons can not be explained by mitochondrial Ca^{2+} overload.

It is also possible that the enhanced glutamate response was the consequence of the partial mitochondrial depolarization produced by DOG. To exclude this possibility, the experiments illustrated in figure 7 were performed. Mitochondria were partially depolarized with a small concentration of the uncoupler dinitrophenol (DNP, 10-12 μM) that mimicked the extent of the DOG-induced depolarization. Then, glutamate was added

into DNP-containing solution. DNP slightly but not significantly increased the percentage of the cells with large mitochondrial and $[Ca^{2+}]_c$ responses, and clearly did not recapitulate the actions of DOG (Figure 7).

It has been shown by others (Drejer at al, 1985; Longuemare et al. 1994; Silver et al. 1997; Tekkok et al. 1999) that hypoxia or hypoglycemia may induce the release of endogenous excitatory amino acids. In our experiments blockade of NMDA receptors by the reversible antagonist memantine (100 μM) during incubation of the cells with DOG did not prevent the increase in sensitivity to glutamate (Data not shown). There is also evidence that a blocker of Na^+ channels tetrodotoxin (TTX) had a protective effect against ischemia (Zeevalk and Nicklas 1991; Vornov et al. 1994; Lynch et al. 1995). However, adding 1 μM TTX to the media during DOG treatment did not alter the change in sensitivity to glutamate (Data not shown).

Selective inhibition of glycolytic ATP synthesis.

Suppression of glycolysis by removal of glucose (or substitution of glucose for DOG) leads not only to the reduction of glycolytic energy production, but also to the decrease of respiration by the depletion of pyruvate, a mitochondrial energy substrate. To determine the specific contribution of glycolytic ATP synthesis 10 mM of pyruvate (with or without of 10 mM lactate) was added to the glucose-free, DOG-containing solution. Addition of the substrates fully prevented the DOG-induced potentiation of neuronal responses to glutamate (Figure 8). This indicates that mitocondrial ATP synthesis can support calcium homeostasis when glycolysis is inhibited. This observation is consistent with the findings of Castilho et al. (1998) in cerebellar granule cells. If the

effects of pyruvate result from supporting respiration, then the blockade of the ATP-synthase by oligomycin should prevent the actions of pyruvate. Figure 9 shows that an addition of 10 μM oligomycin into the glucose-free medium containing pyruvate and lactate fully abolished the protective action of substrates. Because the combination of hypoglycemic medium with an ATP-synthase inhibitor induced a release of endogenous excitatory amino acids and cell swelling (not shown), in these experiments oligomycin was used in the presence of a reversible inhibitor of NMDA receptors (memantine, 100 μM).

Interestingly, adding pyruvate to cells during DOG exposure was notably more protective than adding pyruvate after DOG treatment. In this paradigm, neurons were incubated with DOG for 1h, then washed with HBSS containing 5 mM pyruvate for 25 minutes and finally exposed to glutamate in the presence of pyruvate. Addition of substrate after DOG treatment increased the number of neurons with a small response from 6.5% in DOG without pyruvate to 15.7% (addition of pyruvate *during* DOG treatment increased the number of neurons with small response to 49%). The time to maximum of mitochondrial depolarization increased from 165±23 seconds in DOG alone to 344±29 seconds when DOG was washed out with pyruvate (when pyruvate was added during DOG treatment, this time was increased to 474±28 seconds).

Selective inhibition of mitochondrial ATP synthesis.

We next investigated the consequences of inhibiting mitochondrial ATP synthesis while glycolytic ATP production remained intact. We blocked mitochondrial ATP-synthase using the specific inhbitor oligomycin. Cells were incubated with oligomycin

for 1 h, then washed with HBSS for 20-25 min, after which simultaneous measurements of $[Ca^{2+}]_c$ and $\Delta\Psi_m$ were made. Sample traces and summary data from these experiments are presented in figure 10. DOG treatment was performed in parallel experiments to provide controls within batches of cells. The inhibition of mitochondrial ATP synthesis did not result in the sensitization of responses to glutamate to the same extent as DOG. The difference is not likely to be attributable to the duration of drug action, because oligomycin is usually considered to be irreversible. The data shown in figure 10 indicate that under conditions when glycolysis remained intact, inhibition of mitochondrial ATP synthesis does not increase the sensitivity of neurons to glutamate. This result is in agreement with data Budd & Nicholls (1996a) according to which blockade of mitochondrial ATP synthesis by oligomycin had no effect on the delayed calcium deregulation in the cultured cerebellar granule cells.

Discussion

In this study we investigated the impact of the inhibition of glycolytic and/or mitochondrial ATP synthesis on the sensitivity of neurons to glutamate. Our studies show that an up-stream inhibitor of glycolysis profoundly enhances the sensitivity of neurons to glutamate, as measured by the impairment of neuronal Ca^{2+} homeostasis and mitochondrial depolarization. However, our studies also show that supporting either glycolysis or respiration alone is sufficient to maintain neurons at their normal level of sensitivity. This suggests that neurons in primary culture are different from neurons in the intact brain in that glycolysis may be sufficient to maintain normal Ca^{2+} homeostasis functions.

Glutamate-induced neuronal Ca^{2+} overload leads to neurodegeneration, and a number of studies have concluded that neuronal Ca^{2+} overload and excitotoxicity correlate with mitochondrial dysfunction. Collapse of $\Delta\Psi_m$ should lead to cellular ATP depletion due to an inhibition of mitochondrial ATP synthesis and rapid hydrolysis of cytoplasmic ATP (Budd and Nicholls 1996, a). In this work we pre-incubated the cells with the agents that inhibit glycolytic and/or mitochondrial ATP synthesis and then examined the response of the neurons to glutamate concentrations that do not cause $[Ca^{2+}]_c$ dysregulation in the majority of cells. We have established that an inhibitor of glycolysis DOG greatly enhances the sensitivity of neurons to glutamate. The same concentrations of glutamate, which induced only a small and reversible increase in $[Ca^{2+}]_c$ and mitochondrial depolarization in control cells, after DOG treatment produced a large and irreversible increase in $[Ca^{2+}]_c$ and immediately collapsed $\Delta\Psi_m$. Of special interest is that this effect of DOG was very stable, the cells did not restore they normal

susceptibility to glutamate even after 150 minutes of washout with glucose-containing medium. This is presumably because DOG is phosphorylated and then trapped in neurons (Chi et al. 1987). Blockade of glycolysis leads not only to reduction of ATP synthesis through the glycolytic pathway, but also decreases the supply of energy substrates to mitochondria. To separate the two effects, DOG was used in combination with pyruvate. It has been demonstrated by others that pyruvate protects neurons against glucose deprivation induced toxicity by rescuing the cellular energy charge (Cox et al. 1989; Izumi at al 1994; Matsumoto et al. 1994; Eimerl et al. 1995; Ruiz et al. 1998; Maus et al. 1999; Lee et al. 2001). We have found that pyruvate prevented the effects of DOG on glutamate-induced changes in $[Ca^{2+}]_c$ and $\Delta\Psi_m$, indicating that under conditions of intact respiration, blockade of glycolytic ATP synthesis is not enough to increase neuronal sensitivity to glutamate. To produce a selective blockade of mitochondrial ATP synthesis we used oligomycin. Like selective inhibition of glycolytic ATP synthesis by a combined application of DOG and pyruvate, the oligomycin-induced blockade of mitochondrial ATP synthesis failed to enhance the $[Ca^{2+}]_c$ and $\Delta\Psi_m$ responses to glutamate. In contrast, an addition of oligomycin to the DOG plus pyruvate solution abolished the protective effect of pyruvate (see figure 9) strongly supporting our conclusion that pyruvate protects neurons from a deterioration of Ca^{2+} homeostasis due to maintaining of mitochondrial ATP synthesis but not simply due to restoration of respiration required for generation of mitochondrial potential. These data show that both glycolysis and oxidative phosphorylation have the capacity to support normal cell function.

There are a large number of studies that have showed that neurotoxicity induced by hypoxia, hypoglycemia or oxygen-glucose deprivation is mediated by activation of NMDA receptors through the action of endogenously released glutamate (Novelli et al. 1988; Facci et al. 1990; Goldberg and Choi 1993; Mattson et al. 1993a; Cheng et al. 1993; Maus et al. 1999). This raises the possibility that the sensitization effect is the result of glutamate exposure during the period of hypoglycemia. However, in our experiments addition of NMDA-receptors blocker to the DOG-containing medium did not have a protective effect, suggesting that mechanisms other then stimulation of NMDA receptors contribute to sensitization of the glutamate response. It is possible that the contribution of released glutamate to the sensitization was minimized by the paucity of astrocytes in our culture preparation. We did not find an additional increase in $[Ca^{2+}]_c$ when DOG was applied against a background of a low glutamate concentration (see figure 5), which provides indirect evidence that DOG does not exert a nonspecific effect on glutamate receptor-gated channels. However, we cannot exclude the possibility that the changes in ion influx could occur through the steps downstream of blockade of glycolysis. We have checked the possibility that the effect of DOG could be related with an increase in mitochondrial Ca^{2+} uptake during the treatment of the cells with DOG. The results shown in figure 6 do not support this hypothesis: the release of Ca^{2+} from mitochondria to the response of FCCP was the same in control cells and in neurons treated with DOG. This also emphasizes the notion that mitochondria in resting cells do not store large quantities of calcium (White and Reynolds, 1997).

There are several reports of DOG-induced increase in sensitivity of neurons to glutamate-mediated injury (Lysko et al, 1989; Cox et al, 1989; Rego et al, 1999). Our

results correspond to the data of Cox et al (1989) who showed that lower concentrations of glutamate becomes toxic in cerebellar cultured neurons deprived of glucose. The authors proposed that glucose deprivation leads to depolarization of neurons, relieving of Mg^{2+}-block of the NMDA channels, which promotes ion influx and glutamate toxicity. However, the direct measurement of membrane potential of CA1 hippocampal neurons has shown that 15-20 minutes application of free-glucose, DOG (10 mM) containing medium produced hyperpolarization of neuronal membrane (Zhao et al, 1997). These data are at variance with the hypothesis that DOG-induced increase in sensitivity of neurons to glutamate is due to relief of Mg^{2+}-block of the NMDA channels and the concomitant increase of Ca^{2+} influx into the cytosol.

There are also several reports suggesting that ATP generated from different sources can be preferentially used by the different cellular structures. Thus, it has been reported that the Na-pump may be fueled preferentially by glycolytically generated ATP (Campbell & Paul 1992; Raffin et al. 1992). It has also been suggested by Silver at al. (1997) that this preference is due to a specific requirement for ATP by ATPase imposed by local conditions, which is fulfilled better by glycolysis. Association of some glycolytic pathway enzymes with the plasma membrane and its proteins (Knull 1978) could provide a structural basis for such a 'compartmentalization'. Our experiments did not explicitly demonstrate that one of the cellular sources of ATP production is more important that other for a maintain of Ca^{2+} homeostasis.

The correlation between neuronal degeneration and ATP content is controversial. On the one hand, there are data showing that the irreversible Ca^{2+} overload and excitotoxicity are accompanied by neuronal ATP depletion (Tsuji at al. 1994; Budd and

Nicholls 1996a, b). However, there is also some evidence that ATP depletion does not necessarily correlates with neuronal death, because neurons can survive while experiencing low levels of ATP (Mattson et al. 1993a, b; Riepe et al. 1994; Marcaida et al. 1995). Perhaps the differences in these data can be explain by the fact that the changes in total cellular [ATP] do not necessarily reflect the changes in the free [ATP] in cellular microdomains (Kennedy at al. 1999). Thus, in MIN6 cells changes in [ATP] in cytosol and beneath the plasma membrane [ATP] after exposure to high $[K^+]$ could be detected, even in the absence of significant changes in the total ATP content. It was reported in the same paper that the dynamics of changes in [ATP] in the different cellular subdomains (in the cytosol, mitochondrial matrix and under the plasma membrane) in response to the elevation in extracellular glucose concentration are not the same. It is reasonable to expect that the changes in [ATP] in the neuronal microdomains are also different from those in the bulk of the cell [ATP]; and perhaps even a moderate decrease in the rate of mitochondrial production of ATP, under condition when glycolytic ATP synthesis is inhibited, can greatly reduce [ATP] under the plasma membrane because of the high ATP consumption by the plasma membrane Na-K and Ca-ATPases. However, the measurements of [ATP] in neuronal microdomains have not been performed.

Other factors responsible for the DOG-induced increase in neuronal sensitivity to glutamate may include increase in free radical production, increase in cytosolic $[Na^+]$ ($[Na^+]_c$), or intracellular acidification. It is also possible that oxygen-glucose deprivation induces an increase in free radical production (Almeida at al. 2002), which could contribute to the increase in sensitivity. An increase in $[Na^+]_c$ when the cellular energy production was limited has been demonstrated (Silver at al. 1997). TTX reduced glucose-

and oxygen-glucose deprivation –induced neuronal injury (Lynch et al. 1995; Nijjar and Belgrave 1997) and substitution of extracellular Na^+ with Li^+ prevented NMDA-induced excitotoxicity in glucose-deprived neurons (Czyz et al. 2002). In our experiments TTX did not have an effect, suggested that Na^+ enter through Na^+ channels did not implicate in the effects of DOG. However, we did not investigate whether Na^+ entering through NMDA channels was increased in DOG-treated cells.

In conclusion, we have shown here that a suppression of both glycolytic and mitochondrial ATP synthesis produced a sustained increase in sensitivity of cultured neurons to glutamate. To maintain the sensitivity of cultured neurons to glutamate at the control level, at least one of the sources of ATP production is required to be intact.

Acknowledgements

We thank Geraldine Kress for preparation of the cell cultures, prof. Boris Khodorov and Dr. Alexandr Surin for helpful discussion. This work was supported by the US Army Neurotoxin Initiative grant DAMD 17-98-1-8627 (IJR) and NICHD grant T32-HD40686 (YYH).

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Figure Legends

Fig. 1. Glutamate-induced changes in $[Ca^{2+}]_c$ and $\Delta\Psi_m$.

Simultaneous measurements of $[Ca^{2+}]_c$ and $\Delta\Psi_m$ in individual 13 DIV cultured cortical neurons were made using fura-2FF and Rh123. Neurons were stimulated with 100 μM (A) and 10 μM (B) glutamate in the presence of 2 μM glycine. The mitochondrial uncoupler FCCP (750 nM) was added in the end of experiments to produce a full depolarization of mitochondria.

Fig. 2. Effect of glucose deprivation on neuronal $[Ca^{2+}]_c$ and $\Delta\Psi_m$.

A: Glutamate-induced changes in $[Ca^{2+}]_c$ and $\Delta\Psi_m$ in 13DIV control neurons; **B**: Enhanced response of the cells to10 μM glutamate after a 50-minutes glucose deprivation (free-glucose buffer with addition of 10 mM DOG); **C, i**: Percentage of the cells with mono- (small mitochondrial depolarization) and biphasic (large mitochondrial depolarization) responses to 10 minutes glutamate application and time of the delay in the development of the secondary phase (time to maximum). Each bar and associated error bar represents the mean ± SEM (n= 3 experiments). **ii**: scheme shows the delay interval between the beginning of glutamate application and the maximum of the secondary mitochondrial depolarization that was compared among control and DOG-treated cells.

Fig. 3. Stable effect of DOG on neuronal response to 8 μM glutamate in 14 DIV cell culture.

A: Simultaneous measurements of $[Ca^{2+}]_c$ and $\Delta\Psi_m$ in control neurons treated with glutamate for 10 min; **B**: Cells were incubated in glucose-free, DOG (10 mM)-containing solution for 1h at 37°C and then washed with standard HBSS for 25 min at room temperature. After that the recording was performed at room temperature with a perfusion of standard HBSS; **C**: Histogram showing that glucose deprivation increased the number of neurons with large mitochondrial depolarization and reduced the time to maximum of depolarization; the time to maximum was calculated as shown in figure 3, C, ii. Each column and associated error bar represents the mean ± SEM (n= 12 experiments).

Fig. 4. Effect of DOG on the neuronal $[Ca^{2+}]_c$ response to a short glutamate stimulus. **A**: Increase in $[Ca^{2+}]_c$ in response to repetitive 1.5 minute glutamate (10 μM, glycine, 2 μM) stimulus in control 12 DIV neurons; **B**: The cells were preincubated for 1 h in glucose-free, DOG (10 mM)-containing medium at 37°C, then washed with standard HBSS for 25 min at room temperature. The records were made at room temperature in the presence of glucose.

Fig. 5. DOG does not enhance the glutamate-induced increase in $[Ca^{2+}]_c$. 20 mM DOG and 7 μM glutamate (in the presence of 2 μM glycine) were applied to 13DIV cortical neurons.

Fig. 6. In resting cells mitochondrial Ca^{2+} content remains unchanged after a prolonged DOG application. 750 nM FCCP was applied to the control 13 DIV neurons (A) and to

the cells pretreated with 10 mM DOG for 1 h with a following 20 minutes washout with normal HBSS (B).

Fig. 7. Simultaneous measurements of glutamate-induced changes in $[Ca^{2+}]_c$ and $\Delta\Psi_m$ in 12 DIV cortical neurons with a partial mitochondrial depolarization.

A: Response of control neurons to 10 μM glutamate; **B**: Application of 10 μM glutamate against of a background of partial mitochondrial depolarization induced by 12 μM DNP; **C**: The records were made after the cells were incubated for 1h in glucose-free, DOG-containing solution and washed with standard HBSS for 30 min; **D**: Summarized data show the proportion of the neurons with mono- and biphasic responses to glutamate and the time to maximum of mitochondrial depolarization (calculated as shown in figure 3, C, ii). Each column and associated error bar represents the mean ± SEM (n= 4 experiments); DNP and Control were not significantly different, *p>0.05.

Fig. 8. Effects of DOG and DOG with pyruvate to glutamate-induced changes in $[Ca^{2+}]_c$ and $\Delta\Psi_m$ in 11 DIV cultured cortical neurons.

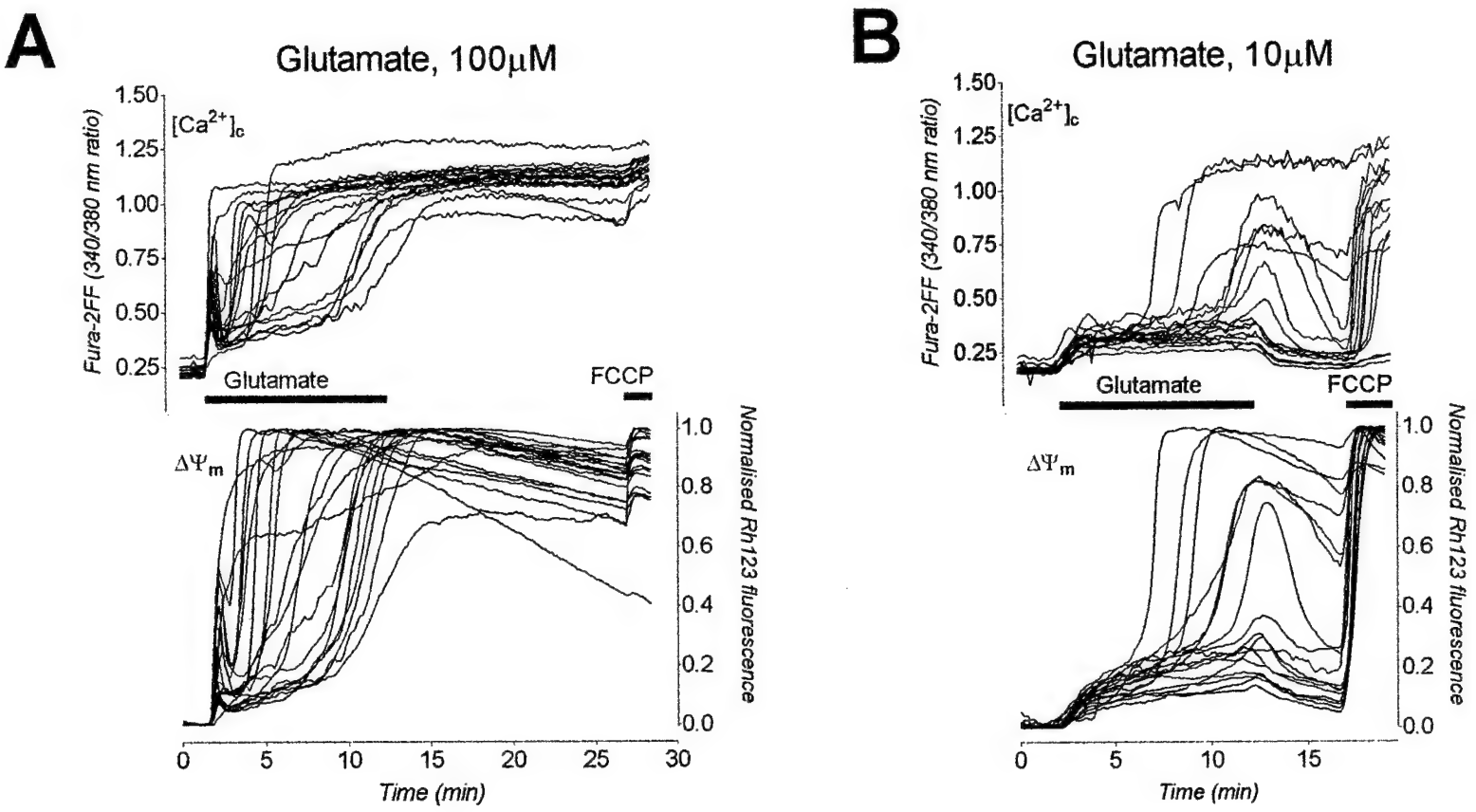
A: Response of control cells to 7 μM glutamate; **B**, **C**: The cells were treated with 10 mM DOG or DOG with 10 mM pyruvate for 60 minutes and washed with normal HBSS for 35 minutes before the recording was performed; **D**: Summarized data of show the proportion of the neurons with small and large responses to glutamate and the time to maximum of mitochondrial depolarization in the cells with biphasic responses. Each column and associated error bar represents the mean ± SEM (n= 8 experiments); DOG+pyruvate and Control were not significantly different, *p>0.05.

Fig. 9. Effects of the blockade of glycolytic or both glycolytic and mitochondrial ATP synthesis on neuronal response to glutamate.

Before the records were made, cultured cortical neurons were treated for 1 h with 10 mM DOG (**B**); 10 mM DOG with 10 mM pyruvate (**C**) or **D**: with a mixture of DOG, pyruvate, 10 μM oligomycin and 100 μM memantine (see explanation in the text), then washed with standard HBSS for 20-25 min at room temperature.

Fig. 10. Effect of blockade of mitochondrial ATP synthesis on neuronal response to glutamate.

For all records, glutamate concentration is 10 μM, FCCP – 750 nM. The records in B-C has been made after 1h incubation of the cells with 10 mM DOG (**B**) or DOG with 10 μM oligomycin (**C**) following 25 minutes washout with standard HBSS. The records of simultaneous measurement of $\Delta\Psi_m$ are not shown. Summarized data presented at the histograms (**D**) were calculated as shown in figure 3. Each column and associated error bar represents the mean ± SEM (n= 4 experiments); Oligomycin and Control were not significantly different, *$p>0.05$.


A
Glutamate, 100μM
[Ca2+]c
Fura-2FF (340/380 nm ratio)
1.50
1.25
1.00
0.75
0.50
0.25
Glutamate
FCCP
ΔΨm
Normalised Rh123 fluorescence
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
Time (min)
B
Glutamate, 10μM
[Ca2+]c
Fura-2FF (340/380 nm ratio)
1.50
1.25
1.00
0.75
0.50
0.25
Glutamate
FCCP
ΔΨm
Normalised Rh123 fluorescence
1.0
0.8
0.6
0.4
0.2
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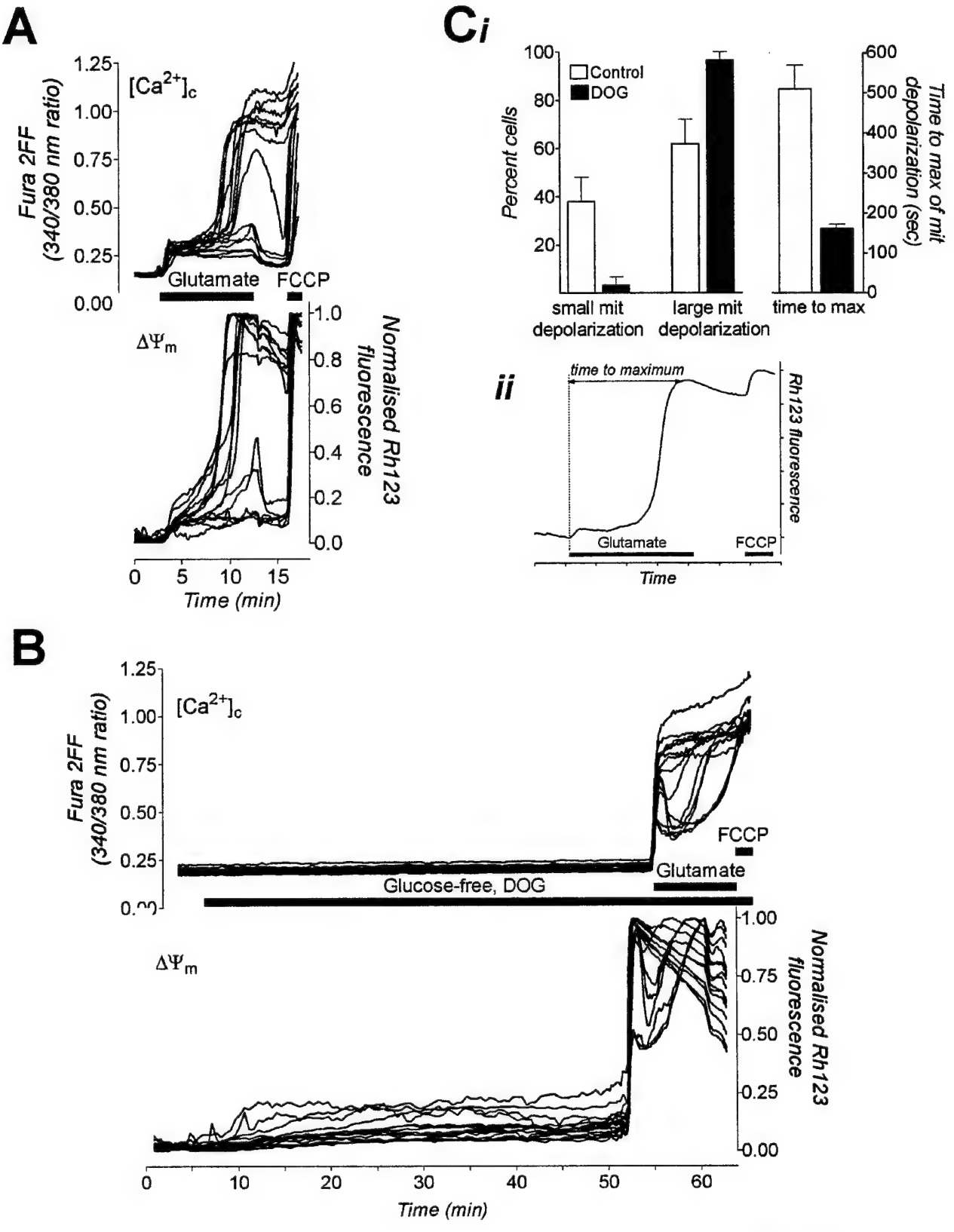
Time (min)

Figure1


A
Fura 2FF (340/380 nm ratio)
[Ca2+]c
Glutamate
FCCP
ΔΨm
Normalised Rh123 fluorescence
Time (min)
Ci
Percent cells
Control
DOG
small mit depolarization
large mit depolarization
time to max
Time to max of mit depolarization (sec)
ii
time to maximum
Rh123 fluorescence
Glutamate
FCCP
Time
B
Fura 2FF (340/380 nm ratio)
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FCCP
Glutamate
Glucose-free, DOG
ΔΨm
Normalised Rh123 fluorescence
Time (min)

Figure 2

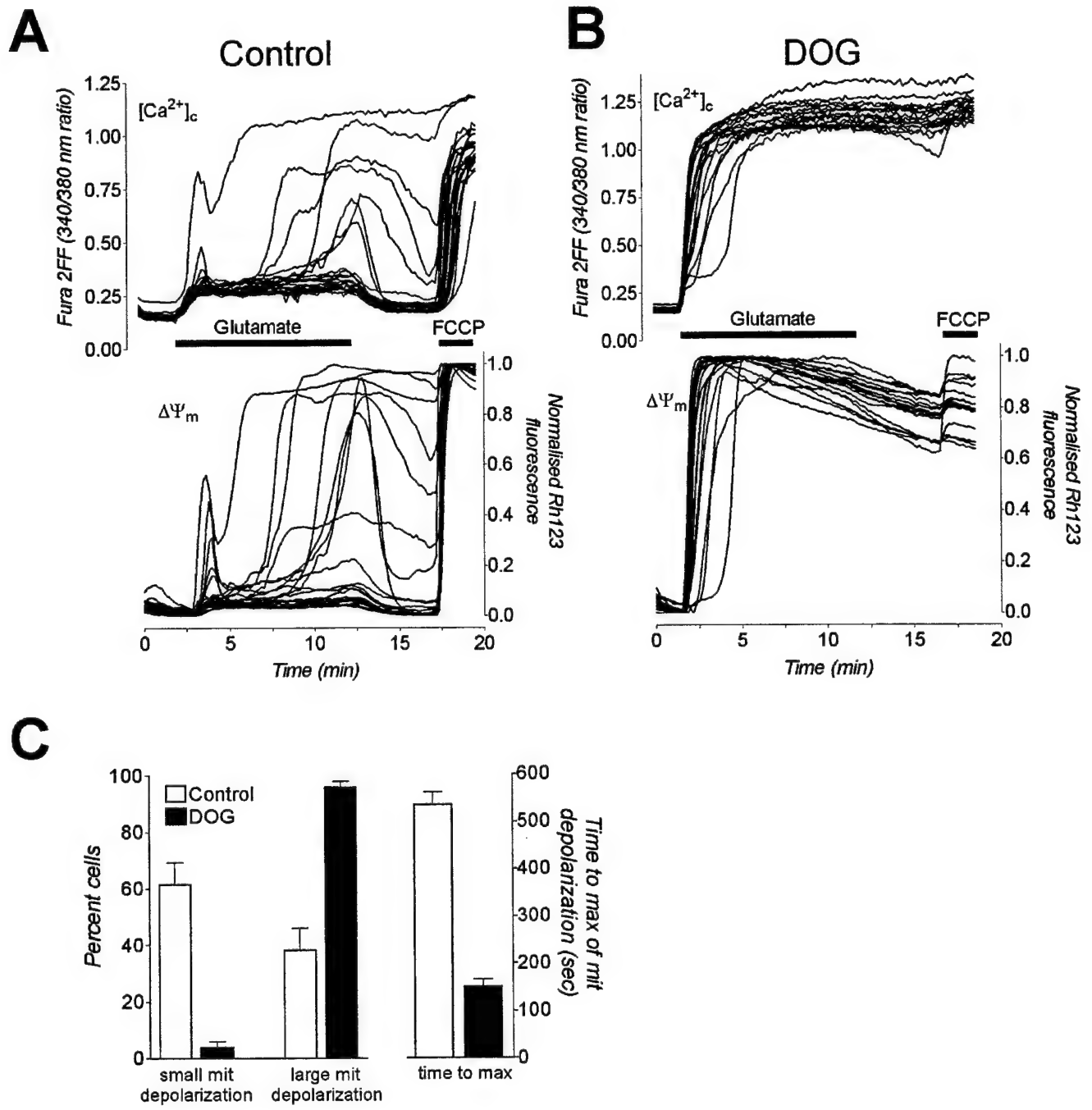
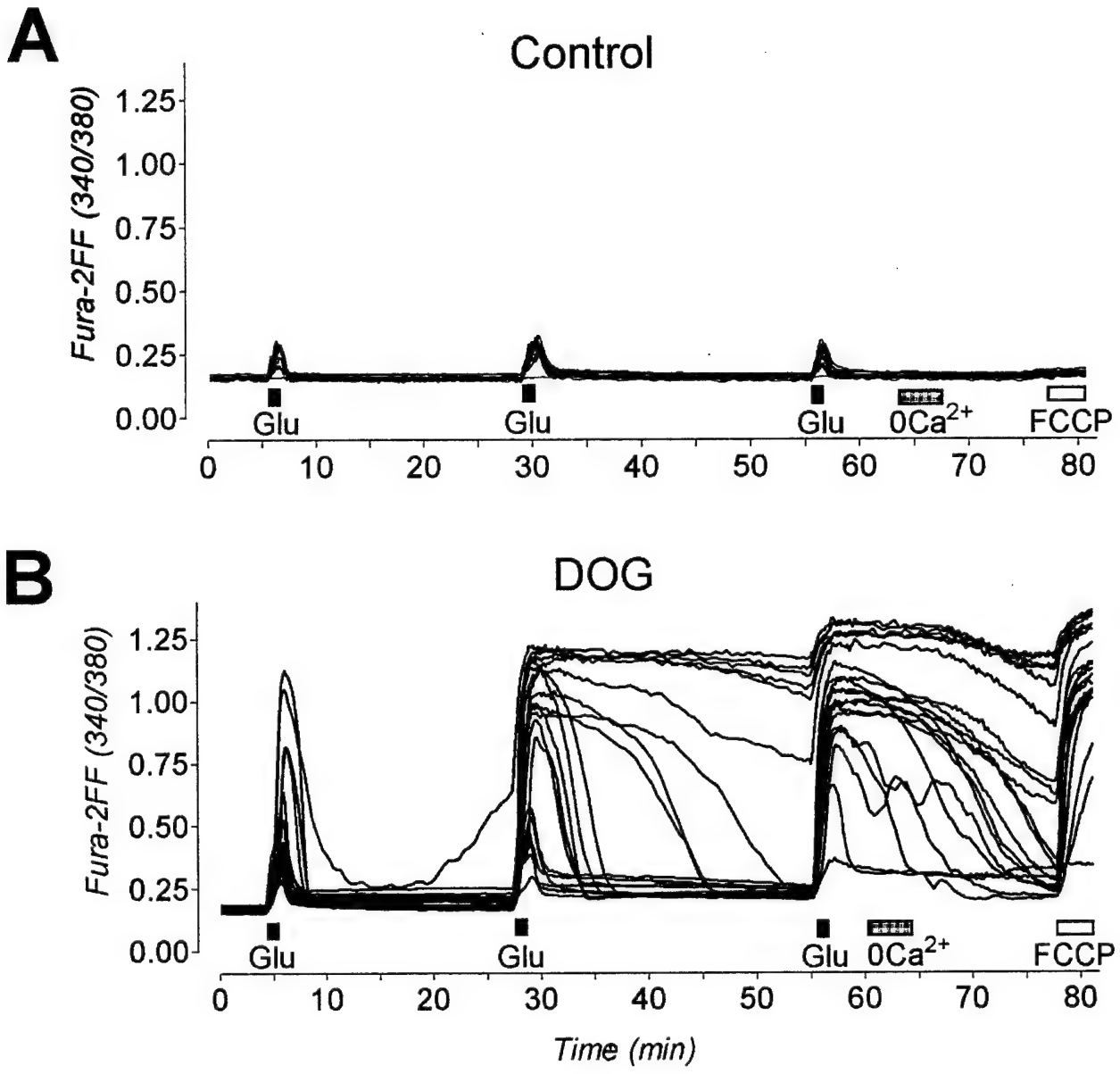

A
Control
B
DOG
[Ca2+]c
Fura 2FF (340/380 nm ratio)
1.25
1.00
0.75
0.50
0.25
0.00
Glutamate
FCCP
ΔΨm
Normalised Rh123 fluorescence
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
Time (min)
C
Control
DOG
Percent cells
100
80
60
40
20
0
Time to max of mit depolarization (sec)
600
500
400
300
200
100
0
small mit depolarization
large mit depolarization
time to max

Figure 3


A
Control
Fura-2FF (340/380)
1.25
1.00
0.75
0.50
0.25
0.00
Glu
Glu
Glu
0Ca2+
FCCP
0
10
20
30
40
50
60
70
80
B
DOG
Fura-2FF (340/380)
1.25
1.00
0.75
0.50
0.25
0.00
Glu
Glu
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0Ca2+
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20
30
40
50
60
70
80
Time (min)

Figure 4

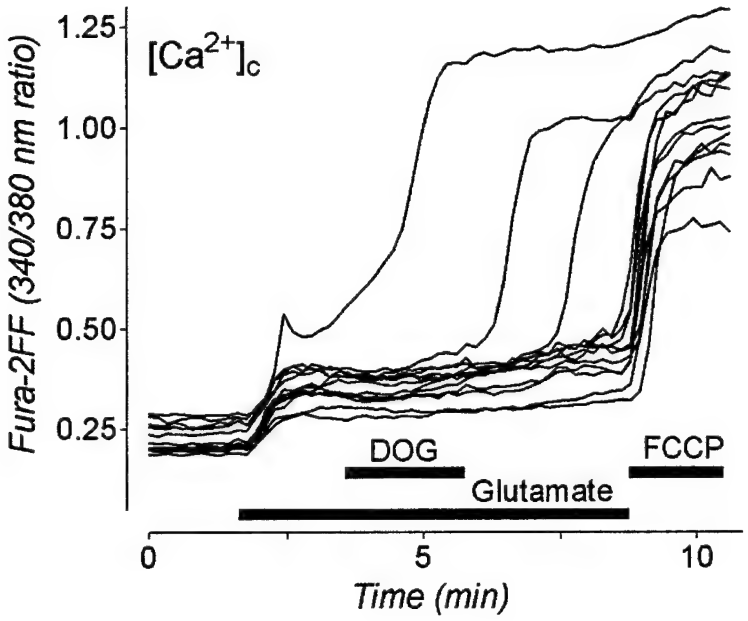
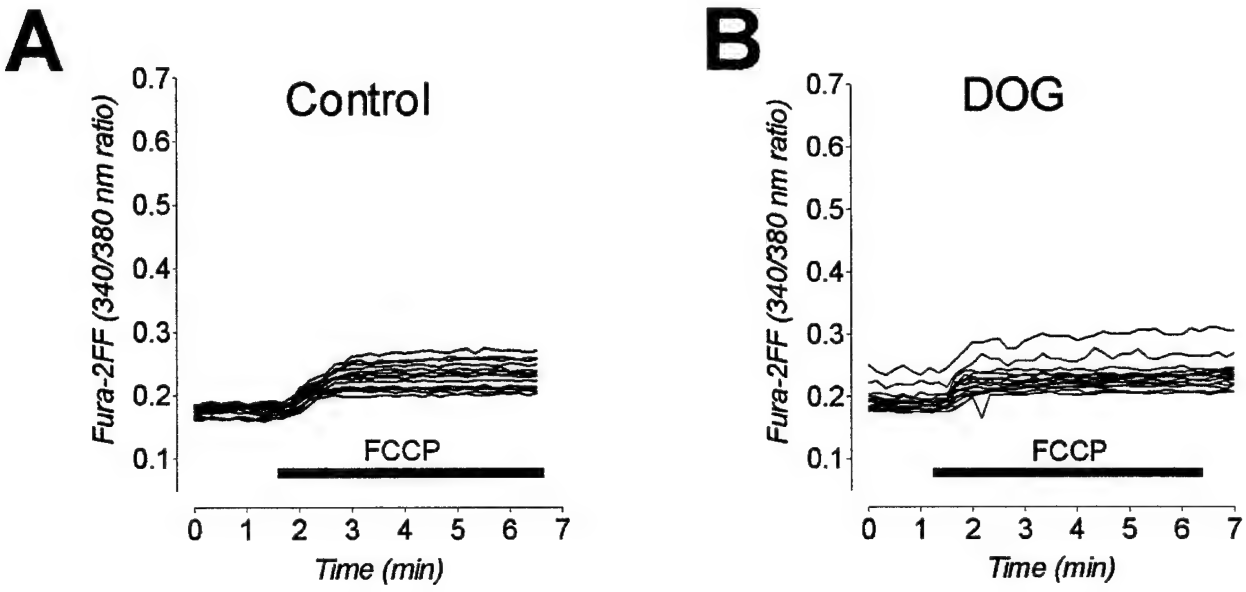

[Ca2+]c
Fura-2FF (340/380 nm ratio)
1.25
1.00
0.75
0.50
0.25
DOG
FCCP
Glutamate
0
5
10
Time (min)

Figure 5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Fura-2FF (340/380 nm ratio)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
FCCP
0 1 2 3 4 5 6 7
Time (min)
B
DOG
Fura-2FF (340/380 nm ratio)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
FCCP
0 1 2 3 4 5 6 7
Time (min)

Figure 6

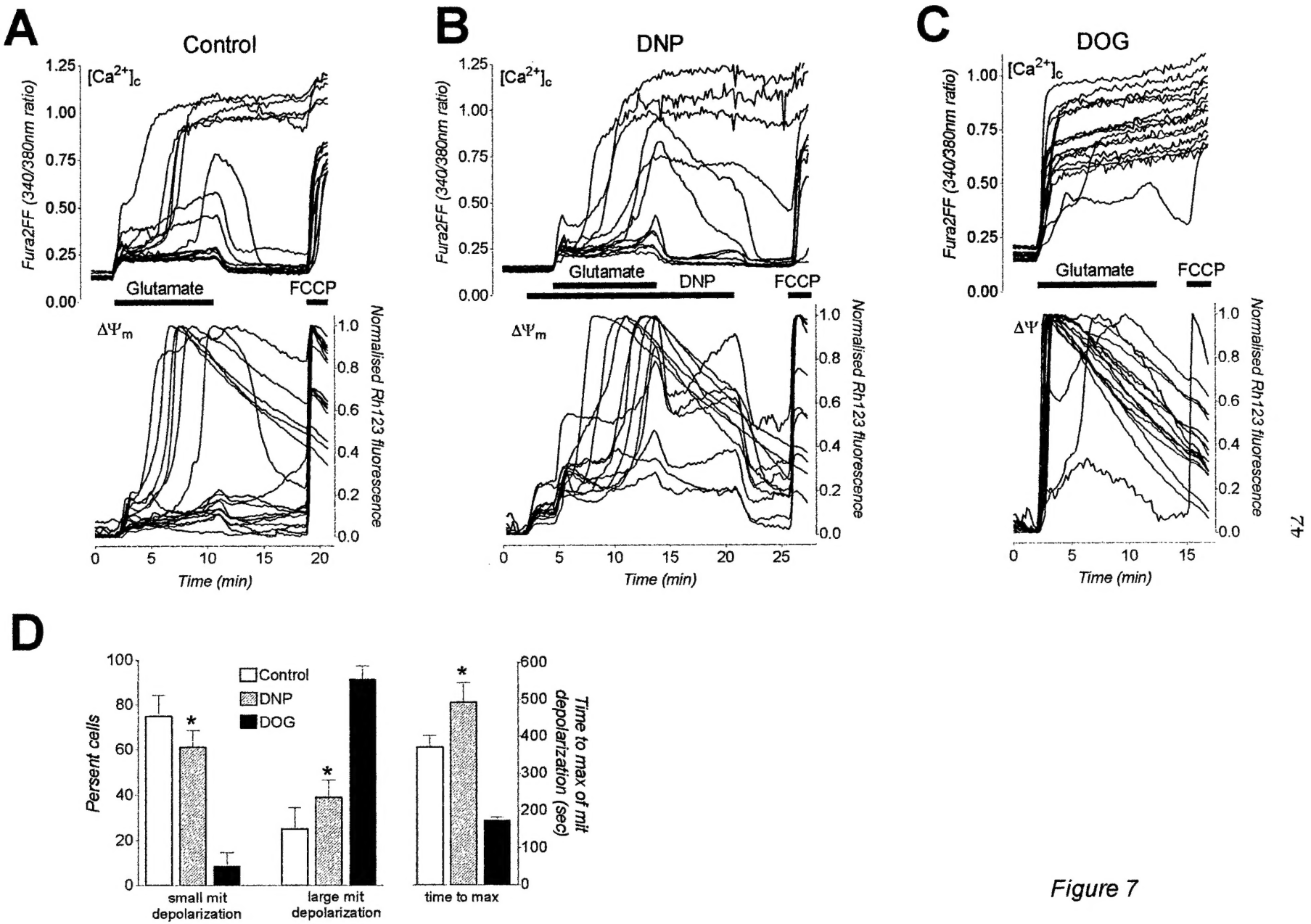
A
Control
B
DNP
C
DOG
[Ca2+]c
Fura2FF (340/380nm ratio)
Glutamate
DNP
FCCP
ΔΨm
Normalised Rh123 fluorescence
Time (min)
D
Control
DNP
DOG
Persent cells
small mit depolarization
large mit depolarization
time to max
Time to max of mit depolarization (sec)

Figure 7

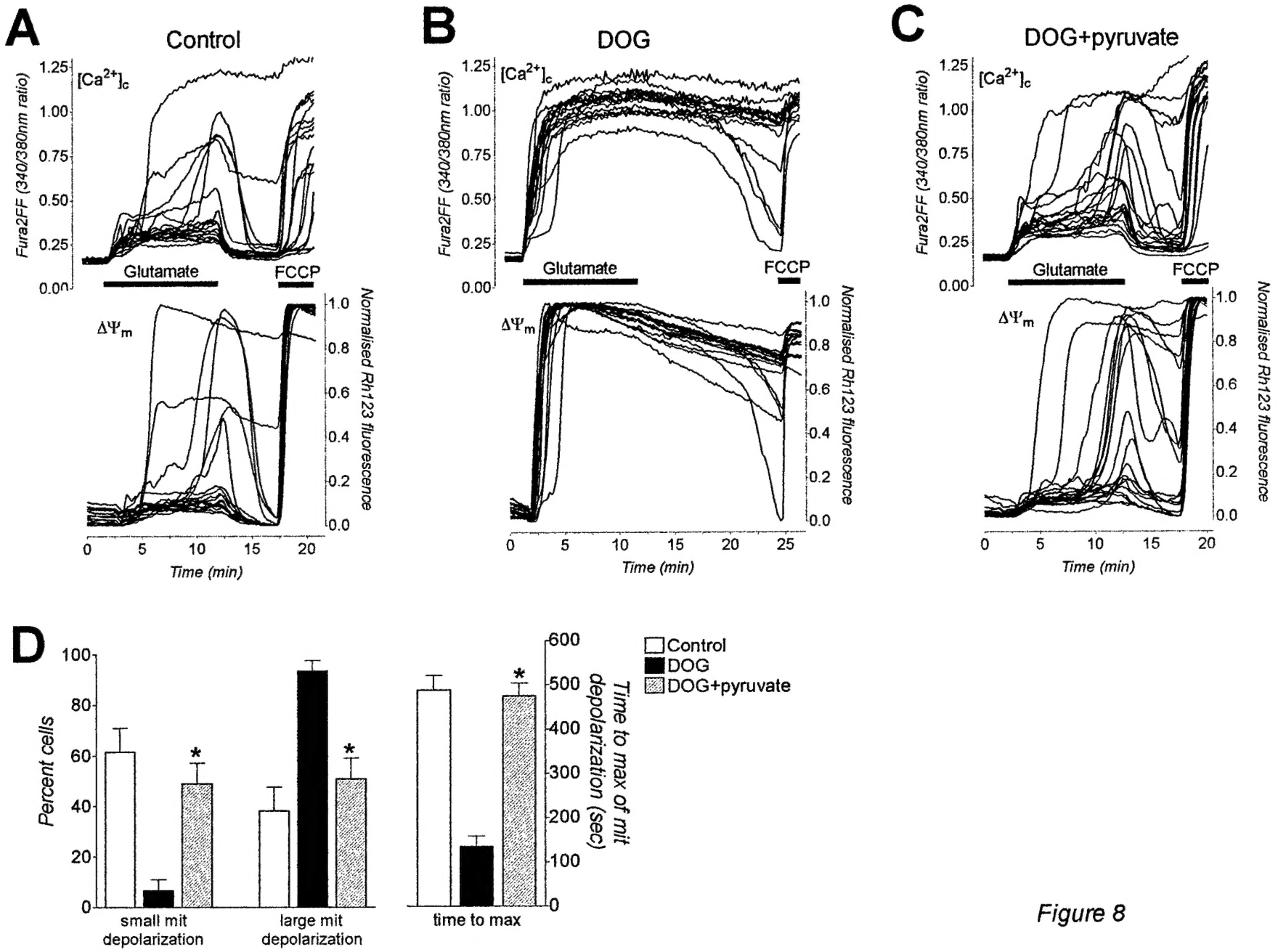
A
Control
B
DOG
C
DOG+pyruvate
Fura2FF (340/380nm ratio)
[Ca2+]c
Glutamate
FCCP
ΔΨm
Normalised Rh123 fluorescence
Time (min)
D
Percent cells
small mit depolarization
large mit depolarization
time to max
Time to max of mit depolarization (sec)
Control
DOG
DOG+pyruvate

Figure 8

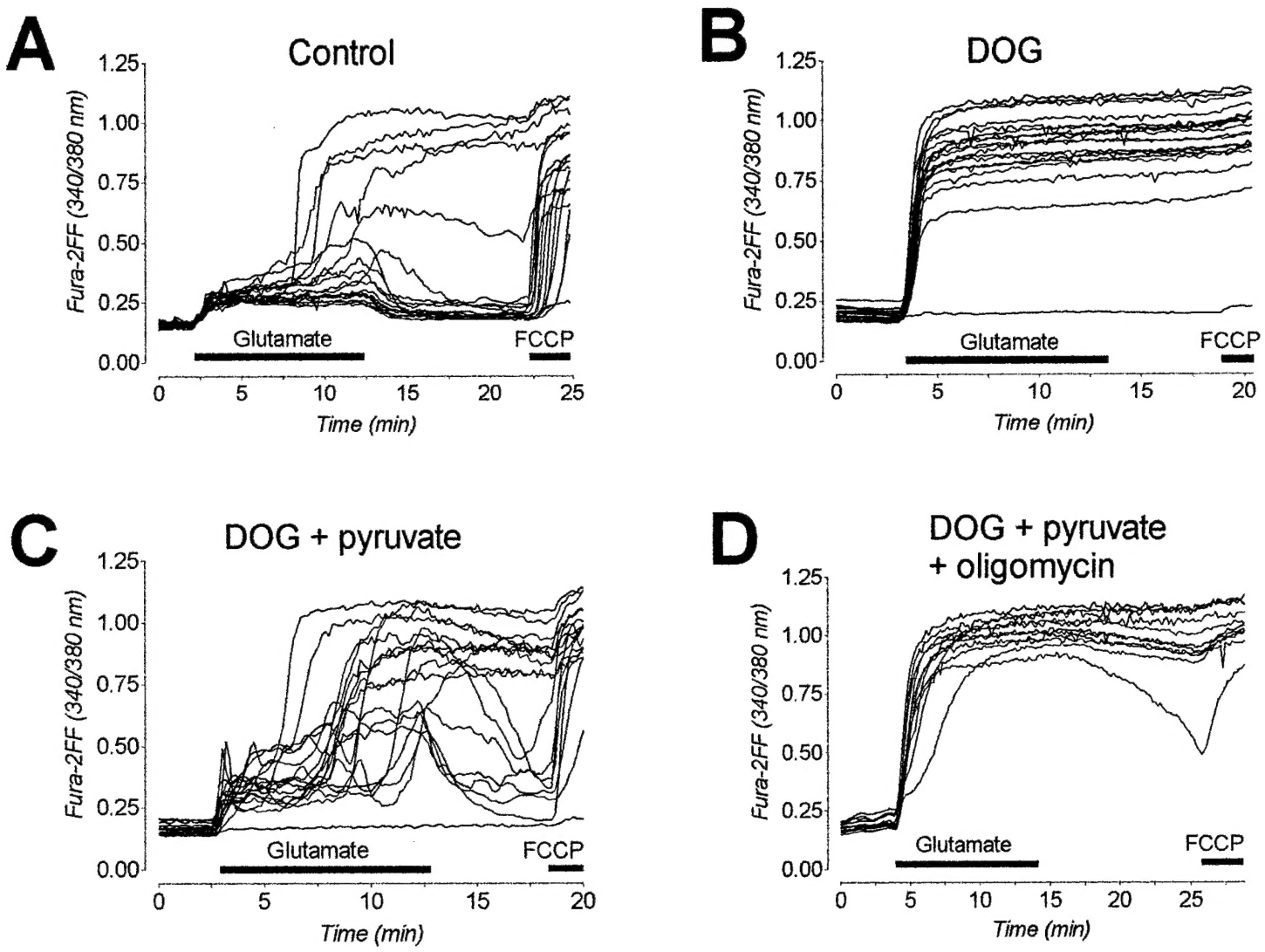
A
Control
B
DOG
C
DOG + pyruvate
D
DOG + pyruvate
+ oligomycin
Fura-2FF (340/380 nm)
Time (min)
Glutamate
FCCP
0.00
0.25
0.50
0.75
1.00
1.25
0
5
10
15
20
25

Figure 9

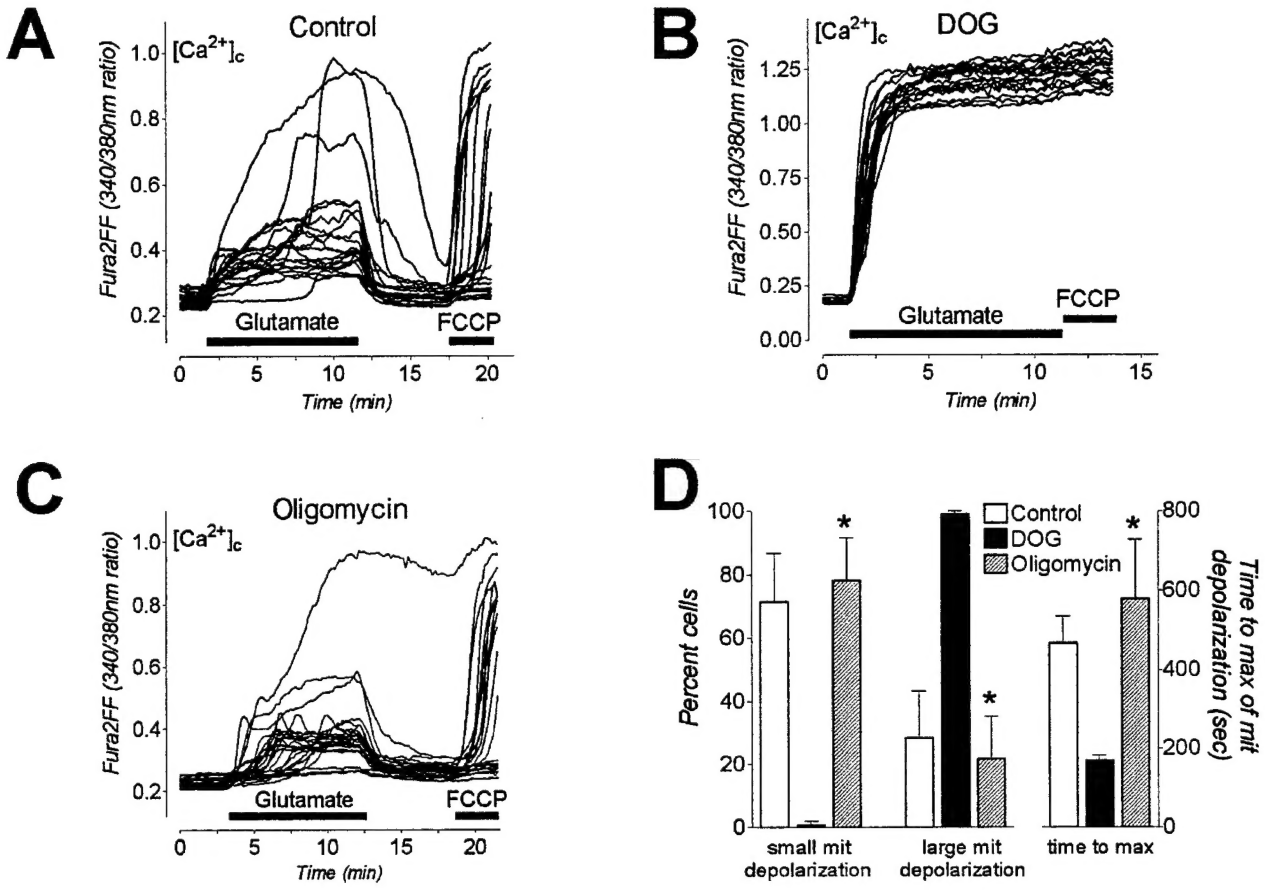
A
Control
[Ca2+]c
Fura2FF (340/380nm ratio)
1.0
0.8
0.6
0.4
0.2
Glutamate
FCCP
0
5
10
15
20
Time (min)
B
DOG
[Ca2+]c
Fura2FF (340/380nm ratio)
1.25
1.00
0.75
0.50
0.25
0.00
Glutamate
FCCP
0
5
10
15
Time (min)
C
Oligomycin
[Ca2+]c
Fura2FF (340/380nm ratio)
1.0
0.8
0.6
0.4
0.2
Glutamate
FCCP
0
5
10
15
20
Time (min)
D
Control
DOG
Oligomycin
Percent cells
100
80
60
40
20
0
800
600
400
200
0
Time to max of mit depolarization (sec)
*
*
*
small mit depolarization
large mit depolarization
time to max

Figure 10